

STUDIES ON THE ACTIVE SITE OF PEPSIN C

by

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ABSTRACT OF THESIS

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Title of Thesis STUDIES ON THE ACTIVE SITE OF PEPSIN C

Pepsin C, one of the minor gastric proteases of the pig, is fairly similar in most of its properties to the major enzyme, pepsin. The most marked difference between the two enzymes is the inability of pepsin C to catalyse the hydrolysis of acetyl-phenylalanyl-diiodotyrosine, a good synthetic substrate for pepsin. It was of interest to try to find which group(s) in pepsin C was involved in the catalytic activity of the enzyme and to make a comparison between this and the active site region of pepsin itself.

The active site of pepsin C was investigated by the technique of 'affinity labelling'. Diazoacetyl norleucine methyl ester was found to inactivate the enzyme very rapidly and irreversibly in the presence of cupric ions at pH values above 4.5. The inactivation was specific, in that one mole of inhibitor was incorporated per mole of enzyme when the enzyme had lost 100% of its activity. Evidence was found for the existence of a binding site for the inhibitor and it was found that a competitive inhibitor protected the enzyme against inactivation by the diazo compound. The irreversible inhibitor did not interact to any great extent with denatured pepsin C, all of which suggests that diazoacetyl norleucine methyl ester inactivates the enzyme by reaction at the active site.

The site of attachment of the inhibitor was investigated using techniques of protein chemistry. It was found that the inhibitor was attached (by an ester link) to the β -carboxyl group of an aspartate residue in the sequence:-

Ile-Val-Asp-Thr-

This sequence was tentatively placed in the longer sequence:-

Leu-Ile-Val-Asp-Thr-Gly-Thr-Ser (Pro, Leu, Ala, Phe)

Use other side if necessary.

Part of this sequence containing an active site residue of pepsin C (Ile-Val-Asp-Thr-Gly-Thr-Ser) is identical to that found for the active site sequence of pepsin by other workers.

Thus, the difference in activities of the minor and major enzymes cannot be explained by a different amino acid sequence around (one of) the reactive group(s). The possibilities that it might be caused by differences in three-dimensional conformation or in amino acid sequence in other parts of the polypeptide chain are discussed.

This thesis has been composed by myself and the work described herein is my own, except where otherwise acknowledged.

TO MY WIFE



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SUMMARY

1. Summary

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Thus, the difference in activities of the minor and major enzymes cannot be explained by a different amino acid sequence around (one of) the reactive group(s). The possibilities that it might be caused by differences in three-dimensional conformation or in amino acid sequence in other parts of the polypeptide chain are discussed.

ABBREVIATIONS AND CONVENTIONS

2. Abbreviations and Conventions

The three letter abbreviations for amino acids are those recommended for use by the Biochemical Journal (Policy of the Journal and Instructions to Authors 1970).

The following abbreviations are used in the text:-

DEAE-cellulose	- Diethylaminoethyl-cellulose
SE-Sephadex	- Sulphoethyl-Sephadex
N-terminal	- Amino terminal
C-terminal	- Carboxyl terminal
[P.U.]	- Proteolytic unit(s)
m[P.U.]	- milliproteolytic unit(s)
FDNB	- 1-fluoro-2,4-dinitrobenzene
DNP-	- 2,4-dinitrophenyl-
DNS-Cl	- 1-dimethylaminonaphthalene-5-sulphonyl chloride
DNS-	- 1-dimethylaminonaphthalene-5-sulphonyl-
APD	- N acetyl-DL-phenylalanyl-L-diiodotyrosine
Z-	- Benzyloxycarbonyl-
Ac-	- Acetyl-
-OMe	- -methyl ester
e.g. Z-Gly-OMe	- Benzyloxycarbonyl-glycine methyl ester
Tris	- 2-amino-2-hydroxymethyl-propane-1, 3-diol
EDTA	- Ethylenediaminetetra-acetate -sodium salt
CALOMe	- Chloroacetyl-L-leucine methyl ester

BALOMe	- Bromoacetyl-L-leucine methyl ester
DNM	- Diazoacetyl-DL-norleucine methyl ester
kV	- kilovolts

INTRODUCTION

3.1 General Introduction.

Enzymes are macromolecules with unique powers of catalytic activity and stereospecificity and the ability to synthesise these catalysts is one of the distinguishing features of living organisms. Thus, it is interesting to appreciate what confers the unique activity and specificity on an enzyme but, before a mechanism of action is assigned to an enzyme, the catalytic reaction must be explained in terms of the intermediates formed, the ways in which bonds are made and broken and any energy changes which may be involved.

After it had been shown (Sumner 1926; Northrop 1939) that enzymic activity was associated with purified proteins, a great deal of research was concentrated on proteins and their constituent amino acids. Techniques were evolved for protein degradation and determination of amino acid sequences. X-Ray crystallography has now become an important tool for the study of protein crystals.

Several early theories on enzyme action were based on the formation of an enzyme-substrate complex during the course of the reaction and this was to explain both the kinetics of the reaction, which showed that the enzyme could be saturated with substrate and a maximum velocity could be reached (Michaelis & Menten 1913), and also the stereospecificity of the reaction (Fischer 1894). Lately, the concept of an enzyme-substrate complex has been shown spectrophotometrically (Chance, Cohen, Jobsis & Schoener 1962) and by its isolation in crystalline form (Yagi 1965).

The demonstration that enzyme catalysed reactions proceed by attachment of the substrate to the enzyme has led to the concept of the 'active site'. This is a certain locus on the enzyme which is responsible for the substrate binding and the catalysis. By modifying the enzyme chemically, it has been shown that certain amino acid residues are essential for the maintenance of these properties, whereas others do not seem to be involved. In comparison with the total number of amino acids in the enzyme, the number situated in the active site is small, but there is a wealth of evidence to indicate that the rest of the enzyme molecule is involved in maintenance of the general conformation of the enzyme. In some cases, it has been shown that changes in structure in regions far removed from the active site can influence the catalytic properties, and the 'induced fit' theory of Koshland (1958) suggested an important role for some so-called secondary amino acids. They may also be involved in regulating the enzyme activity, for example 'allosterism' (Monod, Wyman & Changeux 1965).

While chemical modification studies can show which amino acids are essential for the catalytic activity of the enzyme, it is also necessary to do kinetic and binding studies to decide the roles of the various groups involved. Once adequate structural and kinetic data are obtained, a mechanism of action can be proposed.

3.2. Introduction to the heterogeneity of gastric enzymes.

The ability of gastric juice to digest protein has been known for well over a century and a half and Schwann, in 1836, gave the name pepsin to this ferment, which differed

from the other ferments known at that time by its activity in an acid medium. Sorensen (1907) determined the earliest pH-activity curves for the peptic digestion of albumin and found a maximum around pH 2. In 1930, Northrop obtained a crystalline material from pig gastric mucosa showing similar proteolytic activity and pepsin became one of the first enzymes to be crystallised. The precursor of pepsin in the stomach, pepsinogen, was discovered by Langley (1882).

It is thought that pepsin occurs in the gastric juice of all vertebrates. It has been purified from swine, beef, sheep and chicken and these enzymes all have the same substrate specificity, whereas the specificity of salmon pepsin is different. Pepsins have also been isolated from whale, tuna, shark, halibut and codfish (for a review, see Bovey & Yanari 1960) and preliminary studies show that fish pepsins may have a different specificity from those of warm-blooded animals and are more stable to higher pH values.

Takemura (1909) and Hirayama (1910) were the first to suggest that gastric juice might contain more than one proteolytic enzyme since it had a higher protease activity in the presence of dilute acid than did 'purified' preparations of pepsin. Freudenberg (1940), Buchs (1947) and Taylor (for a review, see Taylor 1962) determined the pH-activity curves of gastric juice and proposed the existence of more than one gastric enzyme.

Michaelis & Davidsohn (1910) subjected a pepsin preparation to electrophoresis at various pH values and found

several components which migrated to the anode and the cathode at pH 1 and 3 but only to the cathode at pH 2. Northrop (1930) also discovered evidence of heterogeneity during his demonstration that his crystalline preparation of enzyme contained activity which was an intrinsic property of the protein itself.

The first indication that there might be four distinct gastric enzymes came from Desreux & Herriott (1939) when they studied the solubility of pepsin in acetate buffer, pH 4.65. Hoch (1950) showed that free boundary electrophoresis at pH 5.9 of crystalline porcine pepsin separated four components, but he did not isolate these and test them for activity.

Caputto, Schultz, Karnes & Wolf (1954) separated on paper two chromatographically distinct but enzymically active components from the gastric juice of fasted humans. These were later separated (Richmond, Tang, Wolf, Trucco & Caputto 1958) by chromatography on Amberlite IRC 50. One of the enzymes was human pepsin and the other was named gastricsin.

Three zymogens (designated I, II and III) were isolated from human fundic mucosa by chromatography on DEAE-cellulose by Seijffers, Segal & Miller (1963a) but one of these, after acidification, gave rise to two enzymes and so would appear to be a mixture (Seijffers et al 1963b). It was indicated in this paper that the four proteases of human gastric juice were present in roughly equal amounts but when pyloric or duodenal mucosa were extracted, only pepsinogen I could be found.

Kushner, Rapp & Burtin (1964), using electrophoresis

in agar gel, showed the existence of four zymogens (designated I, II, III and IV) from human gastric mucosa, but, in contrast to Seijffers et al, found that one zymogen was present in greater amount than the other three.

Hanley & Boyer (1966) found, by starch gel electrophoresis, the existence of three zymogens (designated I, II and III) from swine, human and canine fundic mucosa. It was also found that pyloric mucosal extracts gave weaker reactions than the corresponding fundic mucosa, and, in general, showed the presence of only two zymogens, in contrast to the one reported by Seijffers. The authors suggested that their pyloric cells might have been contaminated with fundic cells and so Seijffers may well be correct.

In 1967, Taylor & Etherington showed the presence of seven zones with proteolytic activity, on electrophoresis of activated human gastric mucosal extracts in agar gel at pH 5.0. In an effort to standardise the confused nomenclature of human pepsins, they suggested that this easily carried out method could be used as a standard test and proposed that their seven zones be called pepsins 1-7, in order of decreasing mobility in this test. Subsequently, they found that pepsin 3 contained a minor zone, 3a, (Etherington & Taylor 1969).

Using this nomenclature, the pepsin I of Seijffers is derived from the pepsinogen II of Hanley and this is probably the same as the pepsinogen III of Kushner since, although they all used different methods of isolation, with Etherington & Taylor's scheme, these all give rise to pepsin 5.

The nomenclature of human pepsins may be confused, but the techniques used to show the apparent heterogeneity can also be criticised. Most authors have relied on staining gels for activity after electrophoresis, which is an easily carried out assay but it might have been an improvement if the supposedly active material had been isolated from their respective bands and tested for activity with more than one substrate (perhaps with a synthetic substrate). Little allowance has been made for possible contamination of zymogen electrophoretograms by active enzyme fragments and similarly contamination of enzyme electrophoretograms by zymogens. Pepsin is also subject to autolytic degradation, which could produce different components and, even in components which have been shown to be active, it is not usually possible to say whether they are autolysis products, whether they are derived from different zymogens or whether their separation is due to binding of different peptides to the same enzyme. Thus, although it is fairly certain that humans have more than one gastric enzyme, exactly how many there are is open to doubt.

Ryle & Porter (1959) found that by chromatographing crude porcine pepsin (which appears to be made by extracting gastric mucosa with dilute sulphuric acid and then using salt fractionation to precipitate the active material) on DEAE-cellulose, two additional enzymes could be isolated. These were called parapepsin I and parapepsin II (later to be pepsin B and pepsin C respectively) in view of their similarity to pepsin in physical, enzymic and chemical properties. These

enzymes differed most markedly from pepsin in their activity towards certain substrates. Pepsin would digest both haemoglobin and the synthetic peptide N-acetyl-L-phenylalanyl-L-diiodotyrosine, whereas pepsin B was inactive in the assay with haemoglobin as substrate and pepsin C would not digest the peptide substrate. It was thought that these enzymes might be intermediates in the pepsinogen \rightarrow pepsin transition, but this was nullified when Ryle (1960) found a distinct zymogen precursor, pepsinogen C, for pepsin C and later, (Ryle 1961; 1965) discovered pepsinogen B (and a fourth minor zymogen, pepsinogen D) in the fundic mucosa of the pig.

From this, it appears that there are (at least) four distinct zymogens in both human and porcine stomachs and each of these gives rise to a distinct enzyme. This is an important conclusion, since it indicates that the minor enzymes cannot be derived from the major pepsins and pepsinogens by autolysis processes.

In 1963, Levchuk and Orekhovitch reported the separation of crude chicken pepsinogen, by chromatography on DEAE-cellulose, into three fractions with potential activity. Since then, Bohak (1969) has shown that chromatography of crude chicken pepsinogen on DEAE-cellulose gives rise to only two fractions with potential activity, and has suggested that these contain the same zymogen contaminated to different extents with impurities which tend to co-chromatograph with it. Thus, it is not clear whether chicken stomachs contain more than one pepsinogen and hence more than one enzyme.

Bovine pepsin was isolated from gastric juice by

Northrop (1933) and solubility studies (Northrop 1930) showed that it was a different protein from porcine pepsin. The specificity (Bergmann & Fruton 1940) and the pH dependence of the activity (Bergmann & Fruton 1941) were studied and both were very similar to those of the porcine enzyme. It was not until 1968 however, that Chow & Kassell reported the isolation of bovine pepsinogen and found evidence for the presence of more than one zymogen in the fundic mucosa, although nothing further was done with these other zymogens. It seems likely that, in parallel with human and porcine gastric mucosa, bovine mucosa also contain more than one zymogen.

Merrett, Bar-Eli & Van Vunakis (1969) reported the isolation of four pepsinogens from the gastric mucosa of the smooth dogfish by chromatography on DEAE-cellulose. Using the order of elution from DEAE-cellulose as the basis for nomenclature (Ryle 1965; Ryle & Hamilton 1966), these were called pepsinogens B, D, A and C respectively. Pepsinogens A and D were very similar to each other, as in the porcine system (Lee & Ryle 1967), while pepsinogen C was different.

It would seem that every species which has been examined does have some heterogeneity with regard to its stomach enzymes. In some species the various enzymes occur to about the same extent, whereas other species, including the pig, have a predominance of one of the enzymes.

3.3. Introduction to studies on porcine pepsin.

Porcine pepsin, called by some authors pepsin A, (and

the zymogen from which it is derived) has undoubtedly been the gastric enzyme on which most studies have been carried out.

Pepsinogen, first crystallised by Herriott (1938) has an isoelectric point of 3.7 and is stable in dilute alkali, pH 7 to 9, whereas pepsin has a very low isoelectric point (close to 1), a very low pH optimum (Perlmann 1955) and is rapidly denatured at pH values above 6. Ultracentrifugation studies have given molecular weight values of 40,000 for pepsinogen and 34,000 for pepsin (Williams & Rajagopalan 1966). Amino acid analysis of crystalline pepsinogen and of pepsin derived from it has been carried out (Rajagopalan, Moore & Stein 1966a) and the results obtained were similar to those previously reported (Van Vunakis & Herriott 1957; Blumenfeld & Perlmann 1959). The amino acid compositions gave molecular weight values of 39,000 and 34,000 for the zymogen and enzyme respectively, in reasonable agreement with the ultracentrifuge data.

End group analysis has shown that both zymogen and enzyme consist of only one chain. Pepsinogen has N-terminal leucine (Van Vunakis & Herriott 1957) whereas pepsin has N-terminal isoleucine. Ong & Perlmann (1968) have reported the sequence of 41 amino acid residues at the N-terminus of pepsinogen and the amino acid composition of this segment agrees very well with the differences in amino acid compositions of pepsinogen and pepsin. This is excellent evidence in support of the theory, first proposed by Herriott, that activation of pepsinogen to the active enzyme is effected by

the release of peptide fragment(s), (including most of the basic amino acid residues) from the N-terminal end of the zymogen. The conversion of pepsinogen into pepsin is catalysed by hydrogen ions and also by pepsin itself, so that the activation proceeds partly autocatalytically below pH 5. In addition to the peptide release involved in the activation, some evidence has been found (Gounaris & Perlmann 1967; Rimón & Perlmann 1968; Grizzuti & Perlmann 1969) that the transition is accompanied by a change in the conformation of the remainder of the molecule.

Heirweigh & Edman (1957) found the sequence:-

Ile-Gly-Asp-Glu

at the N-terminus of pepsin and Trufanov, Kostka, Keil & Sorm (1969) have reported the isolation of a fragment containing 105 residues with N-terminal isoleucine from cyanogen bromide fission plus tryptic cleavage of pepsin, and work is in progress on the sequence of this.

Various workers (Dopheide, Moore & Stein 1967; Vaganova, Levin & Stepanov 1964; Perham & Jones 1967) have found that pepsinogen and pepsin have the same C-terminal sequence, furnishing further evidence that the zymogen → enzyme transition occurs by release of peptides from the N-terminal end, and varying lengths of sequence have been produced for the C-terminus. Pepsin contains only 1 lysine and 2 arginine residues and all of these have been placed in the last 27 residues in the polypeptide chain. This leaves only one basic residue, a histidine, in a sequence of about 300 acidic and neutral amino acids at the N-terminal end of

the enzyme, which may be related to its unusual acid stability and alkali lability.

Pepsin contains three disulphide bonds and sequences have been found for all six half cysteine residues (Keil, Moravsek & Sorm 1967). The single histidine residue has been placed in one of these sequences.

It has been found that both pepsinogen and pepsin contain 1 gram atom of phosphorus per mole of protein. Perlmann (1955; 1958) has proposed the existence of a phosphodiester bond between a serine residue (identified as the site of attachment of the phosphate in the sequence:-

-Glu-Ala-Thr-Ser-Glu-Glu-Leu-

by Stepanov, Vakhitova, Egorov and Avaeva 1965) and another unknown group (possibly the histidine residue?). It was found that the phosphate group was not essential for catalytic activity (Perlmann 1955) and Clement, Rooney, Zakheim & Eastman (1970), studying the pH dependence of the hydrolysis of acetyl-phenylalanyl-tyrosine methyl ester by dephosphorylated pepsin, showed that the phosphate group was not involved in the catalysis. In contrast to Perlmann, however, they found some evidence for the existence of the phosphate as a monoester rather than a diester.

Reports have been published from the laboratories of Stepanov and Keil and Sorm on the isolation of various peptide fragments derived from degradation of pepsin and it is to be hoped that the complete amino acid sequence will be published soon from one of these sources.

Pepsin has been shown to be compact and probably nearly spherical by viscosity and sedimentation studies. The optical rotation and rotatory dispersion constants indicate that there is very little α helical content (Jirgensons 1952; Perlmann 1959) and this is confirmed by the fact that the optical rotation is not changed appreciably in concentrated urea solutions (Kauzmann & Simpson 1953) and enzymic activity is retained (at least when the incubation is done below 20° - Perlmann 1956). Pepsinogen, on the other hand, does have a little α helical content (Perlmann 1963a; Grizzuti & Perlmann 1969), stabilised partially by electrostatic interactions involving the basic peptides removed on activation (Perlmann 1963b).

When pepsin was acidified from pH 7 to 1, little change was noticed in tyrosyl u.v. absorption and so tyrosine-carboxylate ion-dipole bonds (Scheraga 1957) do not seem to be important, although it has been proposed that carboxyl hydrogen bonds are involved in stabilising the conformation of pepsin (Edelhoch 1960) and pepsinogen (Edelhoch, Frattali & Steiner 1965). Kauzmann (1954) has suggested that hydrophobic bonding may be the main stabilising force by which pepsin maintains its conformation.

Although usually considered as a proteolytic enzyme, pepsin has been shown (Lokshina, Orekhovitch & Sklyankina 1964) to have some esterase activity against acetyl-phenylalanyl- β -phenyl lactate, which has a bulky hydrophobic side chain on both sides of the sensitive bond. Although this paper was open to criticism, Inouye & Fruton (1967a) have confirmed that pepsin does have an esterase activity.

Tang (1963) examined the bonds split by pepsin in studies on the primary structure of proteins and found that the enzyme has a preference for leucine, phenylalanine and tyrosine as the amino acids furnishing either the amino or carboxyl groups to the sensitive bond. Schlamowitz & Petersen (1959) found that 'native' protein substrates (the term native must be only relative because of the low pH at which pepsin exerts its activity) were cleaved optimally at pH 2, but after denaturation by acid, alkali or urea, the pH optimum was 3.5.

For hydrolysis of peptides, the amino acid residues must be of the L configuration (Fruton, Bergmann & Anslow 1939) and it appears that acylation of the α amino group increases the susceptibility of peptide substrates to hydrolysis. Many authors have used variations of the basic A-X-Y-B peptide as synthetic substrates for pepsin, where A is an acylating residue, X and Y are phenylalanine or tyrosine and B is either a carboxyl protecting group, e.g. ester or amide, or simply the free carboxyl. These peptides have the disadvantage of limited solubility in aqueous solution and in order to avoid having to add the substrate in an organic solvent (which may have some effect on the catalysis - Tang 1966), Fruton and co-workers have introduced a histidine residue into the peptide, improving the solubility.

A great deal of kinetic data has been obtained for values of k_{cat} and K_M with variations in A, B, X and Y. Authors such as Silver, Denburg & Steffens (1965) and Jackson,

Schlamowitz & Shaw (1965; 1966) have shown that the amino acids on both sides of the bond to be split are involved in the binding of the substrate and they have suggested that hydrophobic bonding is involved in the active site of pepsin (Schlamowitz, Shaw & Jackson 1968). Zeffren & Kaiser (1966) found that, with the peptide substrate acetyl-L-phenylalanyl-L-X where X can be dibromotyrosine, diiodotyrosine or tyrosine, if the values of K_M were taken as an indication of the binding strength, then diiodotyrosine was bound more readily than dibromotyrosine which in turn was bound more readily than the unsubstituted tyrosine. They proposed that either the larger halogenated tyrosines give a better fit in the active site than does the unsubstituted residue or that the polarisable electrons of the halogen substituents interact with some electrophilic centre in the enzyme. Results from competitive inhibition studies (e.g. Inouye & Fruton 1968; Zeffren & Kaiser 1968; Denburg, Nelson & Silver 1968; Hollands, Voynick & Fruton 1969 and Knowles, Sharp & Greenwell 1969) indicate that for hydrolysis of peptide substrates, K_M does approximate K_S - the dissociation constant of the enzyme-substrate complex.

Hollands, Voynick & Fruton (1969) reported that the value of K_M for hydrolysis of Z-His-X-Y-OMe was higher when X-Y was Gly-Phe or Phe-Gly than when Phe-Phe, and so the binding area must be such that it can undergo co-operative interactions with the side chains of both phenylalanines. This work has continued with the substitution of other amino acids in the general Z-His-X-Y-OMe scheme. When X was

isoleucine or valine (Trout & Fruton 1969), there was no hydrolysis and it was found that the K_M values for a variety of amino acids in position X were very similar, but there was a large variation in the values of k_{cat} . The value of k_{cat} for Z-His- β cyclohexyl alanyl-Phe-OMe was one tenth of that for Z-His-Phe-Phe-OMe and so it would seem that the enzyme prefers a planar ring in position X. Z-His-Tyr-Phe-OMe had a lower value of k_{cat} than the Phe-Phe compound but this could be partially overcome by methylating the phenol group. This suggests that the hydroxyl group of the tyrosine in position X may be able to form a hydrogen bond, perhaps with a catalytically important carboxyl in the enzyme-substrate complex. When β cyclohexyl alanine was substituted in position Y, the value of k_{cat} was again much lower. This suggests that the residue in position Y is preferred to be aromatic also, but this time, a tyrosine residue in position Y caused an increase in k_{cat} and methylation of the hydroxyl group caused a decrease in this value, although it was still higher than the values for amino acids with aliphatic side-chains. The authors proposed that, although the apolar side chains of X and Y are important in the rate of hydrolysis of X-Y, additional factors (e.g. stereochemistry) may also be involved.

Jackson et al (1969) studied the hydrolysis of acetyl-phenylalanyl-tyrosine and its corresponding ester and amide at pH 2.0 and 4.5 and found that the K_M values for the non-ionizable substrates did not differ by more than two-fold in this pH range. They also found that the ionizable substrate

was bound much less readily than the non-ionizable ones and the k_{cat} value at pH 4.5 was only 20% of that at pH 2.0. This indicates that there may be a negative charge(s) on the enzyme which repels the negative charge on the substrate at pH 4.5, so that, for hydrolysis of acetyl peptides, the pH must be low enough to protonate the carboxyl group on the substrate before it can be bound and hydrolysed. This was confirmed by Inouye, Voynick, Delpierre & Fruton (1966) who showed that Z-His-Phe-Phe-OEt was hydrolysed more rapidly than the free acid and that the pH optimum for the acid was 3, whereas that for the ester was 4.5. This higher pH optimum for hydrolysis of esters is also interesting in view of the idea (Baker 1951) that the optimal cleavage of acetyl-phenylalanyl-tyrosine at pH 2 represented the action of the enzyme on protein substrates. In view of Schlamowitz & Petersen's observations (page 17), this idea must be corrected, since synthetic substrates ought to resemble the structural situation in denatured rather than 'native' proteins. The lower pH optimum for hydrolysis of native proteins might be explained if the hydrophobic side-chains were in the interior of the protein, with the ionizable side-chains near the surface, so that, for the enzyme to act, the carboxyl groups have to be protonated because of this repulsion effect with negatively charged substrates.

Further evidence that interaction of the A and B groups with the enzyme can affect the catalytic process has been provided by Hollands & Fruton (1968). They found that, for hydrolysis of Z-His-Phe-Phe-OEt, there may be a negatively charged group on the enzyme, close to the imidazole group of

the bound substrate, with an apparent pK_a of 3.8. Inouye & Fruton (1967b) found that if one of the L-phenylalanines was replaced by a D-phenylalanine, the resulting peptide was resistant to peptic hydrolysis. Phe-Phe-OEt was shown to be a competitive inhibitor (Inouye & Fruton 1968) and this might indicate that the Z-His portion of the Z-His-D-Phe-L-Phe-OEt makes little contribution to the binding. Again, the major contribution to binding seems to be provided by the hydrophobic side chains of the phenylalanine residues.

Sacheder & Fruton (1969) have extended their peptide substrate to A-Phe-Phe-B where A can be Z,Z-Gly or Z-Gly-Gly and B is a pyridinium alkyloxy group i.e. the substrates are esters of pyridine alcohols. Changes in A and B had large effects on k_{cat} but little effect on K_M , emphasising the importance of interactions of A and B with enzymic loci, distant from the catalytic site. Such secondary interactions may alter the conformation of the catalytically important group(s) in the enzyme, thus affecting the catalysis.

The effect of pH on the kinetic constants for the hydrolysis of acetyl dipeptides and their esters and amides has been studied by a number of workers. Zeffren & Kaiser (1967), from their work on acetyl-phenylalanyl-dibromotyrosine, proposed pK_a values of 0.75 and 2.67 for the free enzyme (from $\frac{k_{cat}}{K_M}$ values) and 0.89 and 3.44 for the enzyme-substrate complex (from k_{cat} values). They also concluded that the carboxylate group of the substrate (pK_a ca 4) was repelled by an anionic group in the enzyme (as proposed by Jackson et al).

Denburg, Nelson & Silver (1968) found pK_a values of 1.4 and 4.4 for hydrolysis of acetyl-phenylalanyl-tyrosine and its corresponding amide. Lutsenkov, Ginodman & Orekhovitch (1967) found values of 3.0 in the free enzyme and 3.8 and 4.2 in the enzyme-substrate complex with acetyl-phenylalanyl-tyrosine and its ester. Bender & Kezdy (1965) and Clement & Snyder (1966) found a bell-shaped curve for k_{cat} with pK_a values of 1.6 and 3.5 for the hydrolysis of acetyl-phenylalanyl-tyrosine methyl ester. The pK_a values were increased by about 0.4 units in D_2O but the magnitude of k_{cat} was unaffected by D_2O , ruling out a proton transfer in the rate-limiting step.

Clement & Snyder (1968) derived pK_a values of 1.6 and 3.5 from the pH-rate profile of the hydrolysis of acetyl-phenylalanyl-tyrosine methyl ester, acetyl-phenylalanyl-phenylalanine methyl ester and acetyl-tyrosyl-phenylalanine methyl ester. These must, of course, be derived from ionization of groups on the enzyme, since there are no ionizable groups on the substrates. Again, they could find no deuterium oxide solvent effect, thus ruling out a proton transfer in the rate-limiting step. They proposed the involvement of two carboxyl groups in the active site of the enzyme.

Reid & Fahrney (1967) found that cleavage of methyl phenyl sulphite showed no deuterium isotope effect either, but the catalytic mechanism in this case may be different from that of peptide substrates.

However, Hollands & Fruton (1969) determined the kinetic parameters for the hydrolysis of

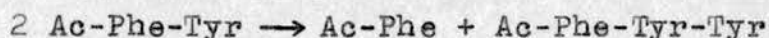
Gly-Gly-Gly-Phe(NO₂)-Phe-OMe in both water and deuterium oxide and found a significant deuterium isotope effect

$\left(\frac{k_{H_2O}}{k_{D_2O}} = 2\right)$. This appears to support the hypothesis that

the catalytic mechanism involves the participation, in the rate-limiting step, of a proton donor (probably an enzyme COOH group) in addition to a COO⁻ group acting as a nucleophile.

It is rather difficult to reconcile these two diametrically opposite schools of thought and obviously further studies are required. It would appear, however, that there are two groups, with pK_a values of around 1 and 4 respectively, involved in the catalytic activity of pepsin and it would seem reasonable to assume that these are carboxyl groups (i.e. one existing as E.CO⁻ and the other as E.COOH).

Pepsin can also catalyse transpeptidation reactions but, in contrast to the other enzymes which have been found to catalyse transpeptidation, it is the amino group and not the carboxyl group which is transferred (Neumann, Levin, Berger & Katchalski 1959). This suggests an amino-enzyme intermediate. It has been shown that the enzyme exerts the same specificity in transpeptidation as in hydrolysis (Mal'tsev, Ginodman, Orekhovitch, Valueva & Akimova 1966) e.g. incubation of pepsin with acetyl-phenylalanyl-tyrosine gave rise to the dipeptide tyrosyl-tyrosine. The following mechanism was postulated:-



and attack on the tripeptide produced Tyr-Tyr.

A free amino group was not necessary for transpeptidation but if the carboxyl group was blocked (e.g. by an amide), there was no transpeptidation. Thus, it may be that attack on the peptide link activates the amino group rather than the carboxyl group and the amino group is transferred to the α carboxyl of another peptide.

Pepsin will also catalyse ^{18}O exchange between water (H_2^{18}O) and the carboxyl group of benzyloxycarbonyl-L-phenylalanine but not of the D isomer nor of the free amino acid (Sharon, Grisaro & Neumann 1962) which might indicate that the carboxyl group must also be activated by the enzyme (Kozlov, Ginodman & Orekhovitch 1962). Transpeptidation between benzyloxycarbonyl-L-phenylalanyl-L-tyrosine and acetyl-L-phenylalanine (Mal'tsev, Ginodman & Orekhovitch 1965) indicated that the new oxygen in the carboxyl group of the product benzyloxycarbonyl-phenylalanine was derived from water and not from the carboxyl group of the acetyl-phenylalanine, as might have been expected if both donor and acceptor reacted directly in a 'four centre exchange' on the enzyme's surface. This suggests that both amino and carboxyl groups are bound to the enzyme.

Bender and Kezdy (1965) and Clement & Snyder (1968) have proposed that the mechanism of action of pepsin involves the formation of a carboxyl anhydride in the enzyme, so that this can react with the substrate to bind both acyl and amino halves.

However, Cornish-Bowden & Knowles (1969) failed to trap an acyl-enzyme intermediate and, although this does not

exclude the possible existence of such an intermediate, it makes the mechanisms of Bender & Kezdy and Clement & Snyder, which proceed through it, rather unlikely. Also, since it has been reported (Shkarenkova, Ginodman, Kozlov & Orekhovitch 1968) that at least one carboxyl group in the active site of pepsin can incorporate ^{18}O very rapidly from H_2^{18}O , it does not seem necessary to have an acyl intermediate to explain the ^{18}O exchange between water (^{18}O) and acyl amino acids.

The mechanism proposed by Jackson et al (1969) is based on the existence of two groups, one on the free enzyme with a pK_a around 4, which must be protonated for the substrate to be bound, and one on the enzyme-substrate complex, with a pK_a of 4.1 when the substrate is ionizable and 4.7 when the substrate is non-ionizable, which must be protonated for hydrolysis to occur. It was assumed in their calculations that these groups were fully protonated at pH 2, which would not seem to be compatible with the data obtained from pH-rate studies by other workers.

Delpierre & Fruton (1965) have suggested that the mechanism by which pepsin exerts its catalytic activity involves an enzymic-carboxylate group (E.COO^-) attacking the carbon atom in the sensitive bond in the peptide R_1CONHR_2 with the formation of 'a tetrahedral intermediate' and the product R_1COOH is released by this intermediate undergoing a 'four centre exchange' reaction, to form an amino-enzyme (E.CONHR_2), (see opposite).

Hydrolysis of the amino-enzyme could be catalysed by the proposed E.COOH , liberating NH_2R_2 (or transpeptidation by amino-transfer to a carboxylic acid) and re-generating

E.COO^- . For this mechanism it was necessary to have the peptide bond protonated before the formation of the tetrahedral intermediate. If the rate of transfer of this proton to the peptide bond, no matter where it comes from, is part of the rate-limiting step, then the rate of enzymic action ought to have shown a deuterium isotope effect.

Studies on pepsin with a number of competitive and non-competitive inhibitors suggest that the first reaction product is liberated after the rate-limiting step. The fact that acetyl-phenylalanine (R_1COOH) is a non-competitive inhibitor (Greenwell & Knowles 1969), whereas phenylalanine methyl ester (NH_2R_2) inhibits competitively, (Inouye et al 1968) indicates that there is an ordered liberation (Cleland 1963) of the products of hydrolysis of R_1CONHR_2 and that R_1COOH is liberated first. However, experiments to observe a 'burst' liberation of R_1COOH have not proved successful (Inouye & Fruton 1967; Cornish-Bowden & Knowles 1969) and evidence for a covalently-linked amino-enzyme has not been found. Since it has been demonstrated that the side-chain of the amino acid donating the NH group to the sensitive bond is important for specificity (Inouye et al 1966; 1967b; Hollands et al 1969), it might be possible to have non-covalent interactions holding the NH_2R_2 product, thus producing the kinetic equivalent of an amino-enzyme.

Thus, the literature is not agreed on the mechanism by which pepsin exerts its catalytic activity but most authors accept the idea of a hydrophobic active site in the enzyme which contains two (and possibly more) carboxyl groups which are intimately involved in the catalytic activity.

A great deal of work has been done to find the groups essential for peptic activity and, particularly, to characterize the carboxyl group(s) purported to be involved in the active site.

The amino groups in pepsin can be modified without any significant loss of activity and the methionine residues can be almost completely alkylated without any loss of activity. When it was found that N-bromosuccinimide (which reacts with methionine and tryptophan residues) inactivated pepsin (Lokshina & Orekhovitch 1964), it was deduced that the reagent was reacting with the tryptophan residues in the enzyme, since the tyrosine residues were not oxidised. When the tyrosine residues are iodinated, diazotised or acetylated, there is a progressive loss in activity (for a review, see Bovey & Yanari 1960), whereas in pepsinogen up to 6 tyrosine residues can be iodinated or diazotised (Neumann & Sharon 1961) without any loss of potential activity. When acetylimidazole was used to acetylate the tyrosine residues in pepsin (Perlmann 1966), it was found that once 9 tyrosine residues had been acetylated, the activity of the enzyme against haemoglobin was reduced to 40% of that of the native enzyme, but the activity against acetyl-DL-phenylalanyl-L-tyrosine was more than doubled. This might possibly be explained by the acetylation protecting the enzyme against inhibition by the DL diastereoisomer.

In an attempt to label the reactive carboxyl group(s), Delpierre & Fruton (1965) introduced diphenyldiazomethane. This was found to inactivate not only pepsin but also

pepsinogen and the reaction was not limited to just one carboxyl group. Since the zymogen was also inactivated, this suggests that either the inactivation was not caused by reaction at the active site or that the zymogen itself was inactivated by reaction of the reagent with some part of the potential active site which was available to the reagent. Some substrates were found to promote the inactivation of pepsin by this compound, indicating that inactivation was not occurring by reaction at the active centre.

All these experiments suffered from lack of specificity by the reagent and other compounds have been developed to overcome this defect. Erlanger, Vratsanos, Wassermann & Cooper (1965; 1966) found that p-bromophenacyl bromide modified a unique aspartate residue (Gross & Morell 1966) in a peptide of composition, Asp,Ser,Glu,Gly₂ with complete loss of peptidase activity and 80% loss of protease activity. It was later found, however, that the diazo analogue, α diazo-p-bromo-acetophenone, modified a different residue with total loss of both activities (Erlanger et al 1967). Since benzyloxycarbonyl-phenylalanine, a non-competitive inhibitor, protected the enzyme against inactivation by p-bromophenacyl bromide, the authors suggested that p-bromophenacyl bromide reacts with a carboxyl group which is close to the active site but which is not essential for catalytic activity, whereas, the diazo analogue reacts with a group directly involved in the catalysis.

Rajagopalan, Moore & Stein (1966b) applied the technique of 'affinity labelling' to pepsin by using diazoacetyl norleucine methyl ester. This rapidly inactivated pepsin in the presence of cupric ions and one mole of inhibitor was incorporated per mole of protein. The inhibitor would not react with denatured pepsin and pepsinogen reacted only very slowly and non-specifically, retaining its full potential activity. This result supports the concept of carboxyl group(s) having a catalytic role in pepsin.

Various authors (Stepanov, Lobareva & Mal'tsev 1967; Hamilton, Spona & Crowell 1967; Ong & Perlmann 1967; Kozlov, Ginodman & Orekhovitch 1967) have since reported the inactivation of pepsin by a number of diazocompounds and attempts have been made to isolate the amino acid sequence containing the modified residue. Fry, Kim, Spona & Hamilton (1968), using 1-diazo-4-phenylbutan-2-one as inhibitor found the sequence:-

Ile-Val-Asp-Thr

around the uniquely modified aspartate residue. Stepanov & Vaganova (1968) using N-diazoacetyl-N'-2,4 dinitro-phenylethylenediamine, reported a

Val-Asp

sequence containing the label. Bayliss, Knowles & Wybrandt (1969), using diazoacetyl phenylalanine methyl ester, found a labelled aspartate in the sequence:-

Ile-Val-Asp-Thr-Gly-Thr-Ser

It seems likely that the same essential carboxyl group is involved in these three studies.

Thus, it would appear that the β carboxyl group of an aspartate residue in the sequence

Ile-Val-Asp-Thr-Gly-Thr-Ser

provides (one of) the necessary carboxyl group(s) for the catalytic activity of pepsin.

3.4. Introduction to porcine pepsin C.

Porcine pepsin C, first isolated by Ryle & Porter (1959), has been shown to have physicochemical properties similar to those of the major enzyme, pepsin (Ryle 1960a; Ryle & Hamilton 1966). Pepsin C, like pepsin, catalyses amino-transfer transpeptidation (Ryle 1960b), so furnishing further evidence of the similarity between the two enzymes. Its amino acid composition and that of its corresponding zymogen (Ryle & Hamilton 1966) show them to be slightly more basic proteins than pepsin and pepsinogen, respectively. Pepsin C does not contain any phosphate but the main characteristic of this enzyme is its inability to catalyse the hydrolysis of the peptide acetyl-phenylalanyl-diiodotyrosine.

Thus, it was of interest to try to find out how pepsin C exerts its catalytic activity and to make a comparison with the major enzyme, pepsin.

It was not possible to do the necessary kinetic experiments to determine the nature of the catalytically important group(s) in pepsin C by pH-rate profiles, binding strengths etc. because of the lack of a suitable synthetic substrate. However, since pepsin C has a pH optimum similar to that of pepsin and shows similarity to the major enzyme in

other general properties, it was a reasonable assumption that the catalytic process might be similar to that of pepsin, in involving carboxyl group(s).

This problem had thus to be tackled from the 'affinity labelling' point of view and the determination of the catalytically important group(s) in pepsin C is the subject of this dissertation.

MATERIALS AND METHODS

4. Materials and Methods

4.1 Determination of activity with bovine haemoglobin as substrate

These assays were done according to the method of Ryle & Porter (1959), based on that of Anson (1948).

The bovine haemoglobin enzyme substrate powder was purchased from Armour Pharmaceutical Co. Ltd. 5g of this was dissolved in 100 ml of water and dialysed to remove low molecular weight material, which, if not removed, would raise the blank values during the assay. The dialysed protein was diluted to 200 ml (a final concentration of 2.5% w/v) and filtered through Whatman No.54 filter paper. Thiomersal (5 mg) was added as a preservative.

The assays were made in duplicate at 37° and pH 1.7. The haemoglobin solution was initially acidified with 0.25 volumes of a solution of hydrochloric acid, whose strength was such that the final pH was 1.7. For enzyme or zymogen dissolved in water, as was usually the case, 0.300N-HCl was used.

The acidified haemoglobin was incubated in a water-bath at 37° for 15 minutes, after which denaturation of the haemoglobin was presumed to be complete. Duplicate 0.2 ml samples of enzyme or zymogen were equilibrated to 37° in the bath along with duplicate tubes containing 0.2 ml of water as blanks, and then 1.0 ml of the acidified haemoglobin was added to each. After exactly 10 minutes the reaction was stopped by addition of 5 ml of a 4% (w/v) solution of trichloroacetic acid. The tubes were then left to stand for

5 minutes to ensure complete precipitation of the protein, and the contents were filtered through Whatman No.3 filter paper. The extinctions of the filtrates at 280 nm were read in a Unicam SP 500 spectrophotometer and subtraction of the mean blank value for the mean 'live' value gave a net extinction at 280 nm which could be converted into milliproteolytic units (m[P.U.]) by reference to standard curves prepared according to the method of Ryle (1960).

4.2 Determination of activity by the clotting of milk.

The method followed was similar to that of Ryle (1960). Gayelord Hauser dried skim milk (20g) was stirred to a paste with water and made up to a final volume of 100 ml after the addition of 1 ml of 1M calcium chloride and 2.5 ml of a 4M acetate buffer to give a final concentration of acetic acid of 0.067M and sodium acetate of 0.05M. The final pH was 5.5.

The enzyme solution (0.5 ml) was equilibrated at 37° and 5 ml of the milk solution (previously equilibrated) was added to this. The contents were mixed by stirring with a glass rod, a stop-clock was started and observations were made at 0.1 minute intervals of the film of milk which clung to the side of the tube. The end-point was taken as the time at which floccular material was first observed in this film.

One proteolytic unit, [P.U.]^{Ren}, is defined as the amount of enzyme which would clot 11.0 ml of enzyme-milk mixture in 1 minute at 37°.

4.3 Total nitrogen determinations.

Total nitrogen determinations on the enzyme or zymogen were done by the micro-Kjeldahl technique (Chibnall, Rees & Williams 1943).

Approximately 1 mg of protein in 1 ml of water was digested overnight with 0.2 ml of concentrated sulphuric acid and about 20 mg of 'Kjeldahl Catalyst' obtained from B.D.H. (made by grinding 1 tablet of sodium sulphate containing 0.1g of copper sulphate with 1 tablet of sodium sulphate containing 0.05g of Selenium). The clear digests were then distilled in a Markham Still, the ammonia evolved being trapped in 5 ml of a solution of 1% boric acid in 20% ethanol. This ammonia was titrated against standard hydrochloric acid.

4.4 Gel filtration using Sephadex and Bio-Gel.

Sephadex was purchased from Pharmacia Ltd., Sweden and Bio-Gel from Calbiochem Ltd.

A certain amount of the appropriate product was weighed out, stirred into a slurry with water and then washed on a Buchner funnel on the water pump with, water, 1N-NaOH (and 1N-HCl in the case of ion-exchange Sephadex) and then water until the pH returned to neutrality. Finally, two volumes of the appropriate buffer or solvent were run through and the gel was left to swell in buffer or solvent for at least the length of time recommended by the manufacturers, but more usually overnight. Fine particles were removed by decantation.

Columns were packed as recommended by the manufacturers. Fractions of constant volume were collected in a Locarte, Central or L.K.B. Radi-Rac fraction collector.

4.5 Electrophoresis on paper at high voltages.

Electrophoresis of peptides was carried out by applying the material, either in spots or streaks, to Whatman No.1 chromatography paper and subjecting the paper to electrophoresis in pyridine-acetic acid buffers, pH 2.0, 3.6, or 6.5, under white spirit as coolant, after the method of Michl (1951). The papers were dried in an oven at 60°. A solution of ninhydrin (0.25% w/v in ethanol, containing 0.1% pyridine) was used for the detection of amino acids and peptides on paper.

4.6 Amino terminal determinations using Fluoro dinitrobenzene.

This technique was largely used for determining the amino terminal residue(s) of the enzyme, zymogen or large peptides. To about 0.1 μ mole of material in 1 ml of solvent were added 0.07 ml of a 25% solution of trimethylamine, 2 ml of ethanol and 0.1 ml of FDNB.

This mixture was shaken for 2 hours in the dark, after which the ethanol was removed on a rotary evaporator and the excess of FDNB was extracted with peroxide-free ether, while keeping the mixture alkaline by addition of trimethylamine. The aqueous phase was taken to dryness on the evaporator and re-suspended in 1 ml of 5.7N-HCl. The tube was flushed with nitrogen and heated for 18 hours in an oven at 105°. The hydrolysate obtained was diluted with 1.5 ml of water and extracted three times with ether.

The ethereal solution containing the DNP-amino acid(s) was taken to dryness and excess of dinitrophenol was removed by sublimation (Mills 1952). The extract was applied to No.4 paper treated with phthalate buffer, along with standard markers and the paper was run in the t-amyl alcohol system of Blackburn & Lowther (1951). If identification was not complete in this system, the DNP-amino acid(s) was run in the 1.5M phosphate system of Levy (1954). Quantitation was achieved by eluting the spot(s) of DNP-amino acid(s) with 5 ml of 1% sodium bicarbonate, measuring their extinction at 360 nm and comparing this with the extinction of standard DNP-amino acids, which had been subjected to the same treatment as the unknown sample, to correct for breakdown on hydrolysis.

4.7 Amino terminal determinations using Dimethylaminonaphthalene-sulphonyl chloride.

The method followed was that of Morse & Horecker (1966). It was applied to the determination of the N-terminus of small peptides. The peptide was dissolved in 0.5 ml of 1% sodium bicarbonate and a 3 fold molar excess of DNS-Cl (6 mg/ml in acetone) was added. Finally, 0.5 ml of acetone was added and the mixture was incubated at room temperature for 2 hours. The solvent was removed from the mixture on the evaporator and the residue was re-dissolved in 1 ml of 5.7N-HCl. The tube was flushed with nitrogen and heated for 18 hours at 105°. The hydrolysate was taken to dryness and re-dissolved in 0.1 ml of acetone.

The DNS-amino acid(s) was identified either by thin layer chromatography on KieselGel G, using the solvent systems of Morse & Horecker (1966) or Deyl & Rosmus (1965), or by two dimensional chromatography on polyamide thin layers (Woods & Wang 1967) in the following solvent systems (Saundry & Hartley, 1969):

1. 1.5% aqueous formic acid
2. Benzene:Glacial acetic acid 9:1
3. Ethyl acetate:methanol:acetic acid 20:1:1

Solvent 3 was run in the same direction as solvent 2.

4.8 Dansyl-Edman degradations.

These were done according to the method of Gray (1967). The peptide, after removal of a sample for DNS-Cl treatment, was dissolved in 0.2 ml of 50% aqueous pyridine and 0.1 ml of a 5% solution of phenyl isothiocyanate in pyridine was added. The tube was flushed with nitrogen, covered with Parafilm and incubated for 1 hour at 45°.

Excess of reagent was then removed by sublimation at 60° for 30 minutes and cleavage of the PTC-peptide was achieved by dissolving the residue in 0.2 ml of anhydrous trifluoroacetic acid and incubating for 30 minutes at 45°. The trifluoroacetic acid was removed on the evaporator at 60°, the residue was dissolved in 0.1 ml of water and diphenyl thiourea (and probably the PTH-amino acid) was removed by extracting three times with 1.5 ml of n-butyl acetate.

The peptide, one residue shorter, remained in the aqueous phase.

4.9 Amino acid analysis.

Hydrolysis of proteins, peptides, DNP-peptides and DNS-peptides was carried out in vacuo or under nitrogen for either 24 or 18 hours at 105°.

Samples for amino acid analysis were dried on the rotary evaporator and dissolved in an appropriate volume of 0.2M citrate buffer, pH 2.2. Amino acid analysis was carried out by the accelerated method of Spackman, Moore & Stein (1958) on a Locarte amino acid analyser.

4.10 Terminal analysis by means of carboxypeptidase A and leucine aminopeptidase.

The enzymes were obtained from the Sigma Chemical Co.

a) Carboxypeptidase A.

The method used for digestion with this enzyme was based on that of Ambler (1967). To prepare the enzyme, an aliquot of the suspension bought from Sigma, containing 50 mg/ml of enzyme, was diluted to 1 ml with water and centrifuged at 2,000 x g for 5 minutes. The crystals obtained were suspended in 0.1 ml of an ice-cold 1% solution of sodium bicarbonate. A solution of sodium hydroxide (0.1N) was then added dropwise until the protein dissolved and the pH was brought carefully back to 8.5 with 0.1N-HCl.

The peptide for digestion was dissolved in 0.2M sodium chloride and the pH was adjusted to 8.5 with dilute sodium hydroxide using a Radiometer TTT-1 autotitrator. After removal of a zero time sample, an appropriate amount of the prepared carboxypeptidase solution was added to give, usually, a final enzyme/peptide molar ratio of 1/50, and the

degradation was allowed to proceed at 37° at pH 8.5, the pH being maintained by addition of dilute alkali, controlled by the autotitrator. Samples were removed for amino acid analysis at various time intervals, the reaction being stopped by addition of 0.1 ml of 1N-HCl.

b) Leucine aminopeptidase.

The method followed was that of Light (1967). The peptide was dissolved in 2 ml of 0.05M Tris-HCl, pH 8.5, and 0.2 ml of 0.025M magnesium chloride was added. After withdrawal of the zero time sample, an appropriate amount of leucine aminopeptidase solution was added, to give a final enzyme/peptide ratio of 1/50, and the incubation was allowed to proceed at 37°.

4.11 Detection of radioisotopes.

¹⁴C glycine was obtained from the Radiochemical Centre, Amersham.

Radioactive material was counted on either a Nuclear Enterprises Scintillation Counter, NE 8304, or a Packard Tri-Carb Liquid Scintillation Counter, Model 314 EX, using Nuclear Enterprises' 'Scinstant C' NE 572 in dioxan, or NE 233 in toluene, as the scintillators.

After application to chromatography paper, radioactive material was detected by means of a 'Radioactive Chromatogram Counter' obtained from Baird and Tatlock (London) Ltd. This consists of a bank of 31 Geiger counters with an associated gas flow system.

4.12 Preparation of pepsinogen C.

The extraction and isolation of pepsinogen C from porcine fundic mucosa was done routinely according to Ryle & Hamilton (1966).

I must thank Mr. A.S. Allan and Mr. J. McGowan who prepared the zymogen.

RESULTS

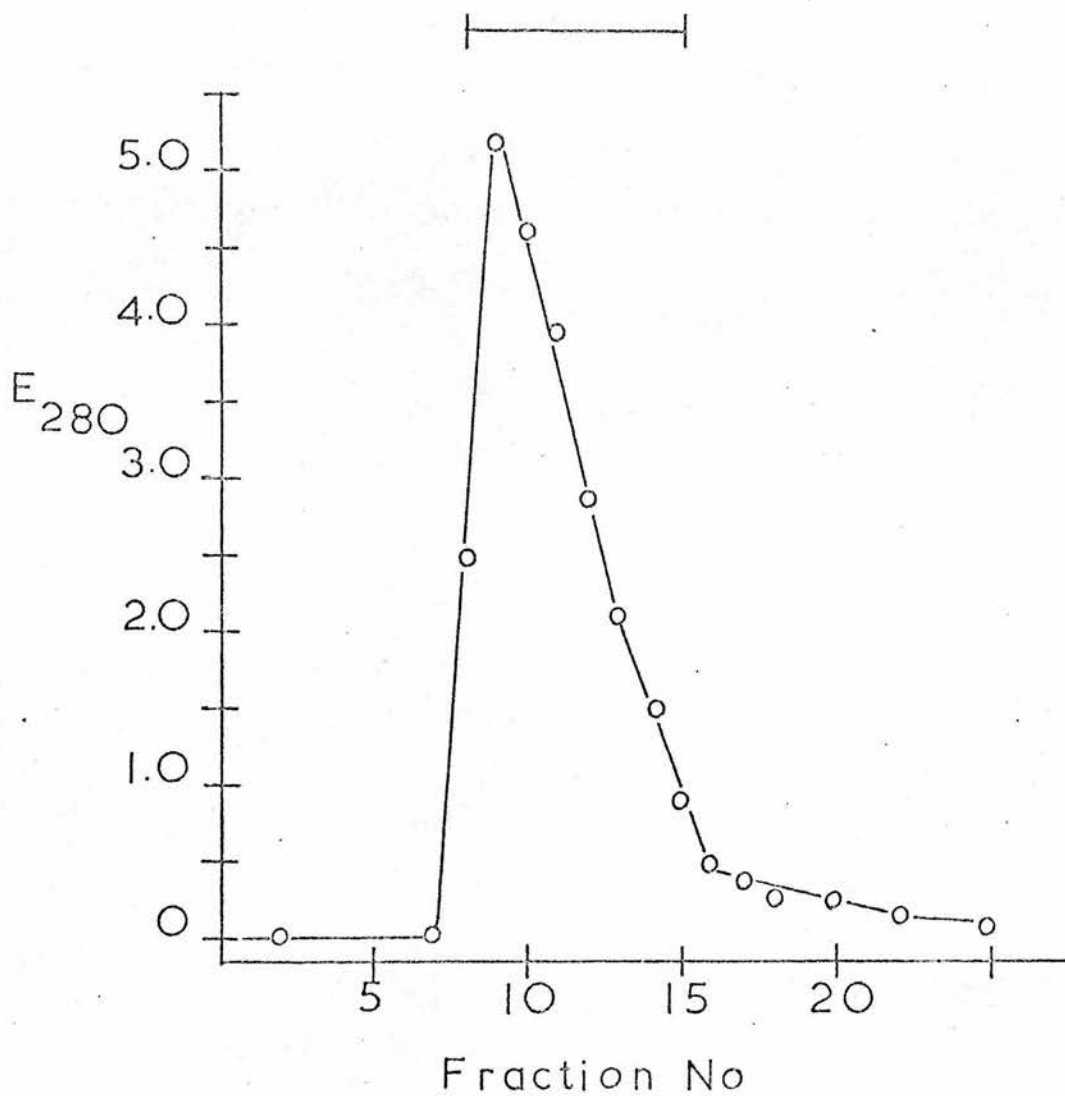


Fig. 1. Chromatography of pH 2 activation mixture from pepsinogen C on SE-Sephadex, C-25, equilibrated with 0.4M acetate buffer, pH 4.4.

5. Preparation of pepsin C from pepsinogen C.

The method followed for this activation was essentially that used by Rajagopalan et al (1966a) for activation of pepsinogen.

Approximately 160 mg of pepsinogen C was dissolved in 8 ml of water. Quadruplicate samples were taken for total nitrogen determination and assays of haemoglobin digesting activity were also done. The specific activity of the zymogen was usually between 0.35 and 0.40 [P.U.]^{Hb}/mg N, compared to that of 0.37 found by Ryle & Hamilton (1966).

The zymogen solution was transferred to a jacketed vessel, which fitted around the electrodes of the auto-titrator, and equilibrated to 14°. The pH of the solution was brought rapidly to 2.0 by addition of a mixture of chloroacetic acid and hydrochloric acid (2N-chloroacetic acid:N-HCl::9:1 v/v) and the material was left to incubate under these conditions for 20 minutes, at which time 4M acetate buffer, pH 5.6, was added to bring the pH to 4.4. After withdrawal of samples of the activated zymogen for determination of the haemoglobin-digesting and milk-clotting activities, the activation mixture was applied to a column of SE-Sephadex, C-25, (2.5 x 50 cm), equilibrated with 0.4M acetate buffer, pH 4.4. The column was operated under gravity at 4° and 5 ml fractions were collected. A typical elution profile is shown in Fig. 1.

After reading the extinctions of the fractions at 280 nm, the fractions containing the protein were pooled and, after adjusting the pH to 5.0 by addition of 2M sodium

acetate, the pooled material was dialysed against distilled water at 4° , changing the water every 4 hours until the material in the dialysis sac became milky. The sac was then opened and the material was freeze-dried.

The activation peptides were eluted from the column by running through 0.4M acetate buffer and then 0.1N-ammonia. Sequence studies on these are in progress (Falla & Ryle, unpublished observations).

The specific activity of the enzyme was found by total nitrogen determinations, haemoglobin and milk-clotting assays. Values around $0.5[\text{P.U.}]^{\text{Hb}}/\text{mg N}$ and $0.20[\text{P.U.}]^{\text{Ren}}/\text{m}[\text{P.U.}]^{\text{Hb}}$ were obtained, compared to those of 0.41 and 0.47 found by Ryle & Hamilton for pepsin C obtained from crude commercial preparations of pepsin by chromatography on DEAE-cellulose and re-chromatography of the pepsin C peak on Sephadex G-100. It was not possible to compare directly the enzymes prepared by the two methods, since more modern commercial preparations do not contain pepsin C.

The haemoglobin-digesting activity of pepsin C appears to be slightly higher than that found previously but there is a large discrepancy in the abilities to clot milk. Since the milk-clotting assay has a subjective end-point and since this assay was not to be used for any of the subsequent work, not too much emphasis was placed on this variation, but it was deemed necessary to check the purity of the pepsin C by other methods.

Pepsin C
Pepsin C (Ryle & Hamilton)

Lys	5.3	4
His	0.9	1
Arg	3.3	4
Asp	27.9	28
Thr	24.5	25
Ser	34.5	35
Glu	41.4	41
Pro	18.0	18
Gly	33.0	32
Ala	19.9	21
Val	19.2	20
Met	2.3	4
Ile	14.1	14
Leu	34.1	34
Tyr	16.7	18
Phe	20.4	21

Table 1. Amino acid composition of pepsin C prepared by chromatography on SE-Sephadex.

5.1 Amino acid analysis of pepsin C.

Samples of the enzyme were hydrolysed in vacuo at 105° for 24 hours and subjected to amino acid analysis. Table 1 shows the values obtained, with those of Ryle & Hamilton for comparison. In the determination of the molar ratios of the amino acids, the results obtained for Asp₂₈ Ile₁₄ and Arg₄ were sufficiently constant and nearly integral to permit the values for these groups to be used to calculate the values for the other amino acids. Values for half cystine are not included because of the failure of this amino acid to separate completely from alanine on the amino acid analyser. The values for threonine and serine were obtained by assuming a 5% and 10% loss respectively.

A close correlation exists between pepsin C prepared by SE-Sephadex and that of Ryle & Hamilton, with the possible exception of an extra lysine residue in the former.

5.2 Determination of the amino-terminus of pepsin C.

This was done using FDNB. The DNP-protein was hydrolysed for 18 hours with and without the addition of the DNP-derivatives previously identified in a qualitative experiment as occupying the N-termini, to obtain correction factors for breakdown on hydrolysis of these DNP-amino acids. Chromatography of the ether-soluble DNP-amino acids in t-amyl alcohol (Blackburn & Lowther 1951) and in the phosphate system of Levy (1954), revealed the presence of 0.6 moles of DNP-serine/mole of enzyme and 0.2 moles of DNP-leucine or isoleucine with smaller amounts of alanine and phenylalanine. This confirms the N-termini found by

Ryle & Porter (1959). It may be that activation of pepsinogen C leaves a product with a slightly heterogeneous N-terminus e.g.

Ile-Ser---

and Ser---

since Ryle & Porter found the material to be monodisperse on ultracentrifugation and starch-gel electrophoresis.

Another explanation, of course, is the presence of a peptide contaminating the enzyme but, since the amino acid composition of the protein showed no evidence of extra amino acids, this seems unlikely, unless pepsin C prepared by both methods contains the same peptide.

5.3 Determination of the carboxyl-terminus of pepsin C (and pepsinogen C).

As a further check on the homogeneity of pepsin C, the C-terminal sequence was studied and compared with that of pepsinogen C. It was necessary to inactivate the enzyme and the potentially active zymogen to avoid contamination by autodigestion.

a) Preparation of carboxymethyl pepsinogen C (CMCg).

The method used was based on that of Crestfield, Moore & Stein (1963a). Pepsinogen C (80 mg) was dissolved in 3 ml of Tris-HCl buffer, pH 8.6, in a screw-capped dark bottle under an atmosphere of nitrogen. Urea (3.6g), 0.3 ml of a solution of EDTA (50 mg/ml) and 0.1 ml of mercaptoethanol were added. The volume was made up to 7.5 ml with water and the bottle, then, was completely filled with a solution 8M in urea and 0.2% in EDTA. A

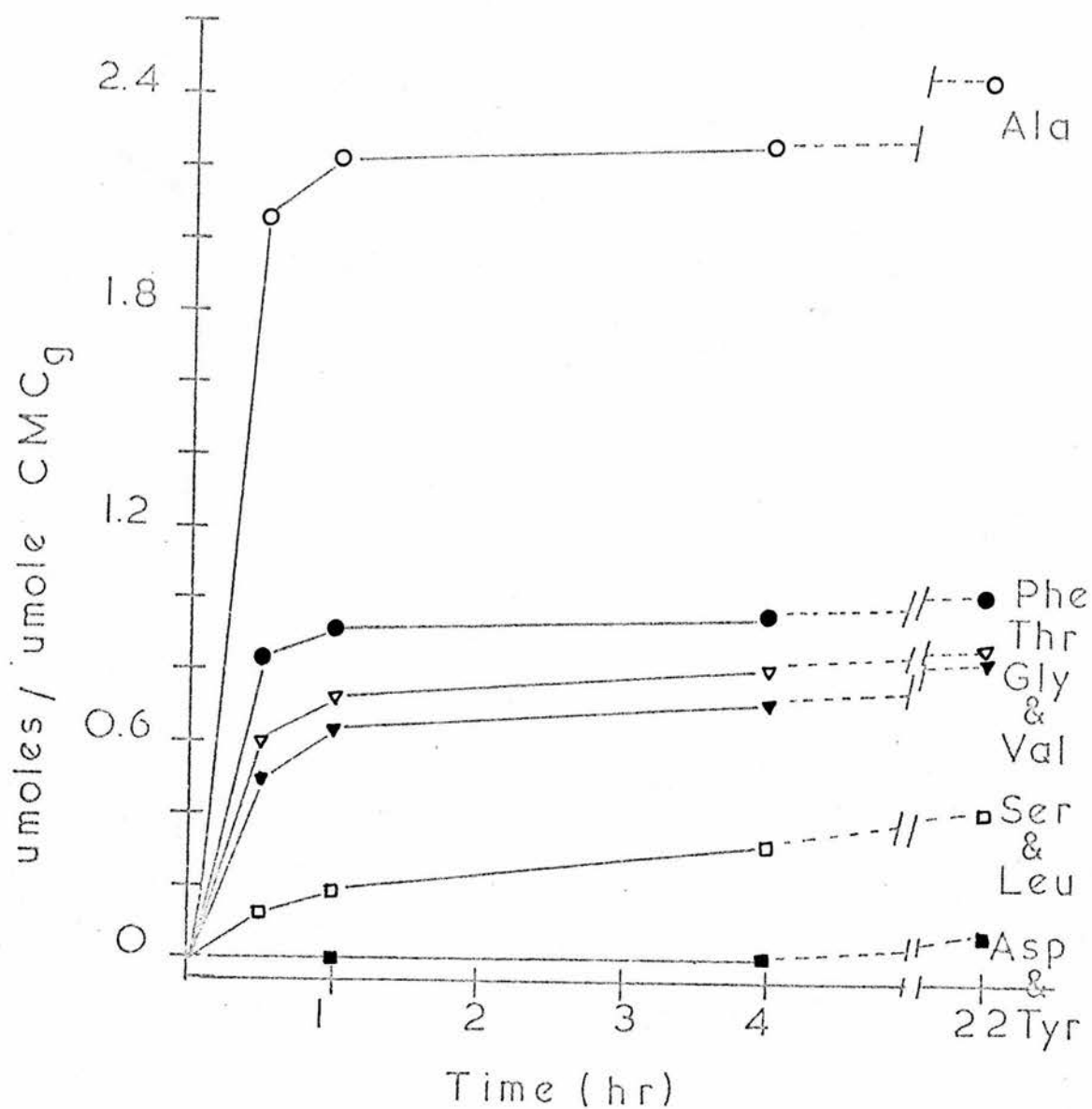


Fig. 2. Time course of the release of amino acids from carboxymethyl pepsinogen C by carboxypeptidase A.

polythene disc was slipped on top to exclude air and the cap was screwed firmly on. This mixture was left to stir for 4 hours at room temperature at which time it was transferred to a 50 ml beaker under an atmosphere of nitrogen and a solution of 0.268g of iodoacetate in 1 ml of 1N-NaOH was added. This mixture was stirred in the dark for 15 minutes and then transferred to an Amicon "Diaflo" ultra-filtration cell, equipped with a UM 20 membrane. The material left in the cell after several cycles of concentration and dilution with water was freeze-dried and the yield obtained was 40 mg. A small sample was hydrolysed and amino acid analysis showed the presence of carboxymethyl cysteine.

b) Digestion with carboxypeptidase A.

The reduced carboxymethylated zymogen was treated with carboxypeptidase A (Section 4.10a) and samples were taken for amino acid analysis at 0, 0.5, 1, 4 and 22 hours. The reaction was stopped by adding 0.1 ml of 1N-HCl (hence the need to inactivate the enzyme and zymogen prior to this) and the samples were left at 0° for 15 minutes. The denatured protein was then centrifuged off. The supernatants were taken to dryness on the rotary evaporator and re-dissolved in an appropriate volume of citrate buffer, pH 2.2, for application to the amino acid analyser. The time course of the release of amino acids is shown in Fig. 2. Alanine (2 moles/mole of protein) was released very quickly, accompanied by 1 mole each of phenylalanine and threonine. This was followed by a slower release of glycine and the

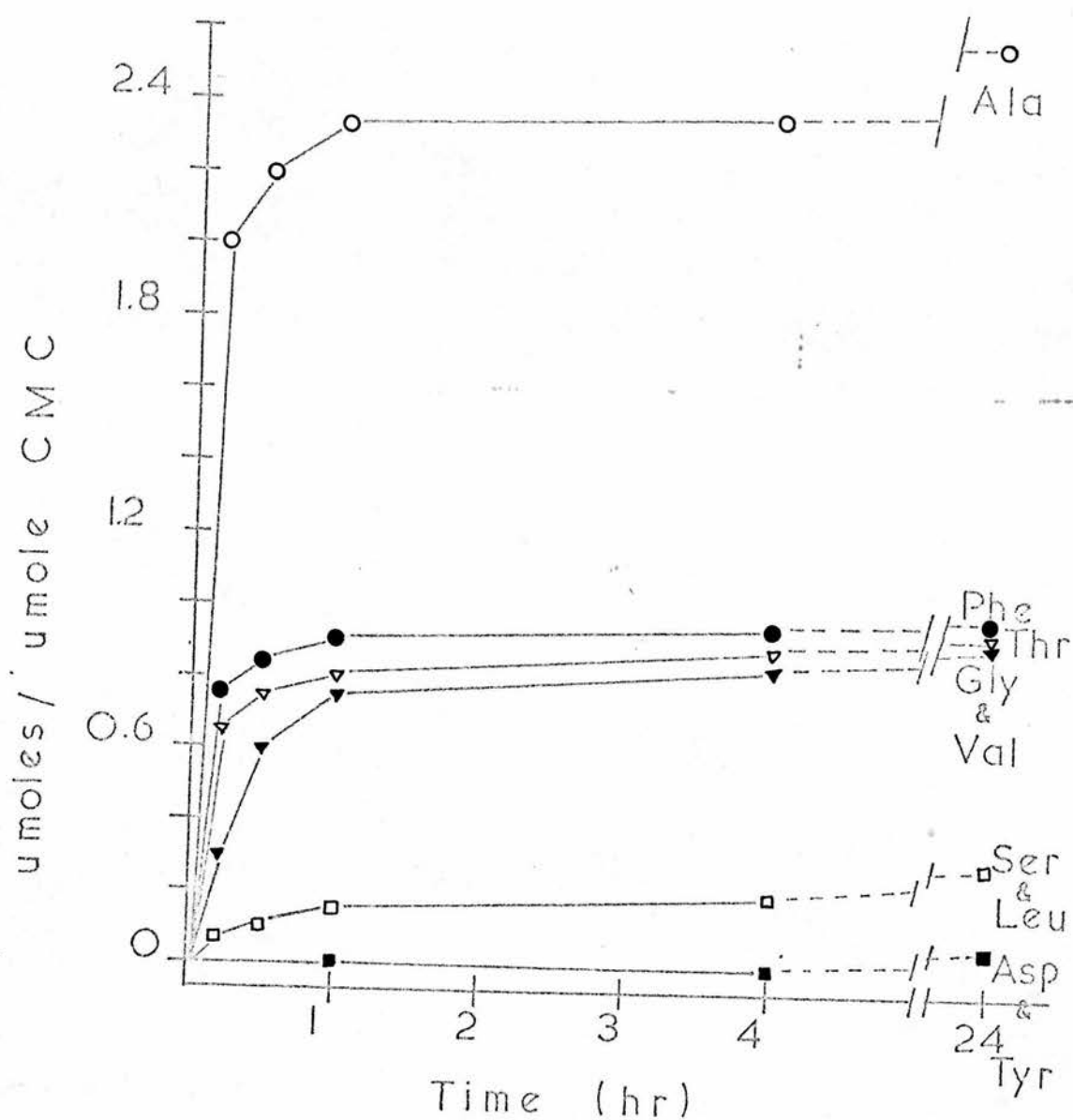


Fig. 3. Time course of the release of amino acids from carboxymethyl pepsin C by carboxypeptidase A.

liberation of valine followed this exactly. Serine was released even more slowly and this was paralleled by the release of leucine and another residue of alanine. Finally, aspartate was very, very slowly liberated and tyrosine was released in parallel with this. Because of the variations in the rates of release of glycine, serine and aspartate by carboxypeptidase A, it can be proposed that the C-terminal composition is:-

(Ala,Leu,Ser)(Val,Gly)(Thr,Phe,Ala,Ala)

c) Preparation of inactive pepsin C.

It was found in a trial experiment that reduction and carboxymethylation did not destroy the enzymic activity quickly enough to prevent autodigestion, so a method based on that of Stepanov & Vaganova (1963) was used. Pepsin C (43 mg) was dissolved in 1 ml of 90% phenol (v/v) and this was left at 20° for 20 minutes. Dry ether was added and the precipitated protein was centrifuged off and dried over sodium hydroxide in vacuo. The yield was 34 mg. In order to compare directly the pepsin C with pepsinogen C, the phenol treated protein was reduced and carboxymethylated as before.

d) Digestion of pepsin C with carboxypeptidase A.

This was carried out as before, samples being taken at 0, 0.2, 0.5, 1, 4 and 24 hours. The time course is shown in Fig. 3. The pattern of release is very similar to that found for pepsinogen C, suggesting a common C-terminal sequence for pepsin C and pepsinogen C.

This test for homogeneity of pepsin C was done on the basis that pepsin C might have had a similar C-terminal sequence to that of pepsin, i.e.

-Pro-Val-Ala (Dopheide, Moore & Stein 1967)

and therefore only alanine is released by carboxypeptidase A. However, more than one amino acid was released but, because of the spacing of the slowly released glycine, serine and aspartate residues in the sequence, more information has been achieved than could have been anticipated.

The fact that both zymogen and enzyme have the same C-terminal composition suggests that pepsin C may be produced from pepsinogen C by activation at the N-terminal end, in common with all the other known inactive precursor → active enzyme transitions.

6. Affinity labelling and pepsin C.

Since no synthetic peptide substrate was available for pepsin C, the nature of the active site had to be investigated by affinity labelling. It was thought that a carboxyl group(s) might be involved in the catalytic mechanism and so a pseudo-substrate was needed which might react with a carboxyl group.

6.1 Attempted inactivation of pepsin C by chloroacetyl-L-leucine methyl ester (CALOMe).

a) Preparation of CALOMe.

Acetylation of leucine was done under Schotten-Bauman conditions.

L-leucine (0.02 moles) was dissolved in 40 ml of 2.5N-NaOH cooled to 0° and 2 ml (0.025 moles) of chloroacetyl-chloride was added portionwise with constant stirring. Once the addition was complete, stirring was continued for 1 hour at 0° and then the solution was acidified by addition of concentrated hydrochloric acid. The material which precipitated was filtered off and re-crystallised by dissolving it in 2.5N-NaOH and re-precipitating with hydrochloric acid. The yield was 1.6g (40% of theoretical), m.p. 132° (literature value 133°).

Esterification was carried out by dissolving the chloroacetyl-leucine in 4 ml of methanol cooled to 0° and adding thionyl chloride (1 ml) dropwise with constant stirring (Brenner & Huber 1953). On completion of the addition, the solution was slowly warmed to 37° and kept at this temperature for 2 hours. The solution was filtered

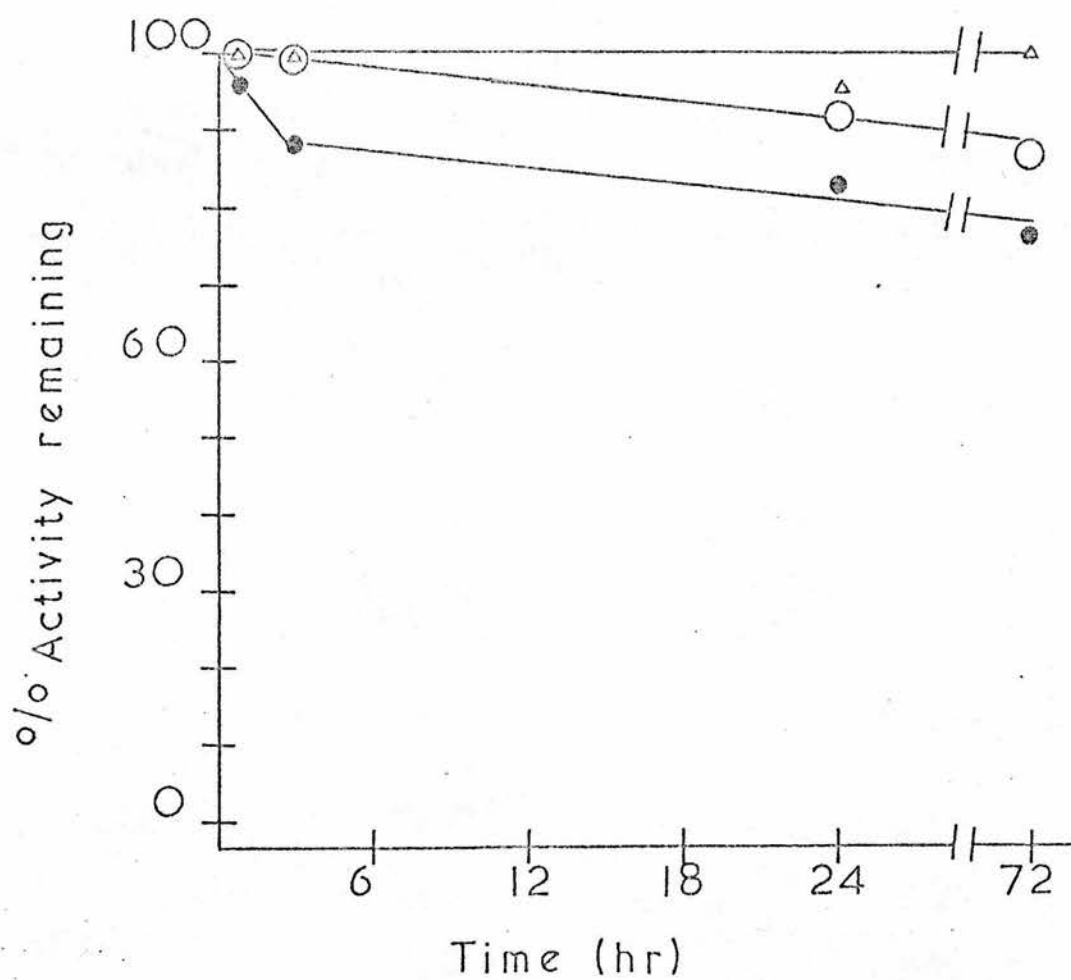


Fig. 4. Inactivation of pepsin C by CALOMe at two pH values.

- △ - control at pH 2
- - pH 2
- - pH 5

through a sintered glass funnel and ether, dried over sodium wire, was added to the filtrate. The precipitate was collected and re-crystallised from methanol and ether. The yield was 0.15g (8.3%) and the m.p. was 152.5°.

b) Attempted inhibition by CALOMe.

This was tried at pH 2 and pH 5 with a molar ratio of CALOMe/enzyme of 220/1. Tubes were set up containing 10 ml of enzyme solution (0.1 mg/ml) in 0.01N-HCl or 0.2M acetate buffer, pH 5.0, and, after withdrawal of zero time samples, 0.1 ml of a solution of CALOMe (20 mg/ml in methanol) was added to each. Incubation was carried out at 20° and, for haemoglobin assay of activity, 0.1 ml samples were taken, diluted to 2 ml with water and 0.2 ml of the diluted solutions were used for the assay. The results (Fig. 4) show that CALOMe was a very poor inhibitor of pepsin C, producing 24% inhibition after 72 hours incubation at pH 2 and even less (13%) at pH 5. The control shows that autodigestion did not seem to be a problem, at this concentration of enzyme at least, and 0.1 ml of methanol did not inhibit the enzyme to any great extent (cf. Tang 1966).

6.2 Attempted inactivation by bromoacetyl-L-leucine methyl ester (BALOMe).

a) Preparation of BALOMe.

This was prepared by a Schotten-Bauman reaction analagous to that for CALOMe using bromoacetyl bromide to acylate the leucine. On acidification of the alkaline incubation mixture no precipitate was observed and so the solution was extracted with ethyl acetate. The ethyl acetate

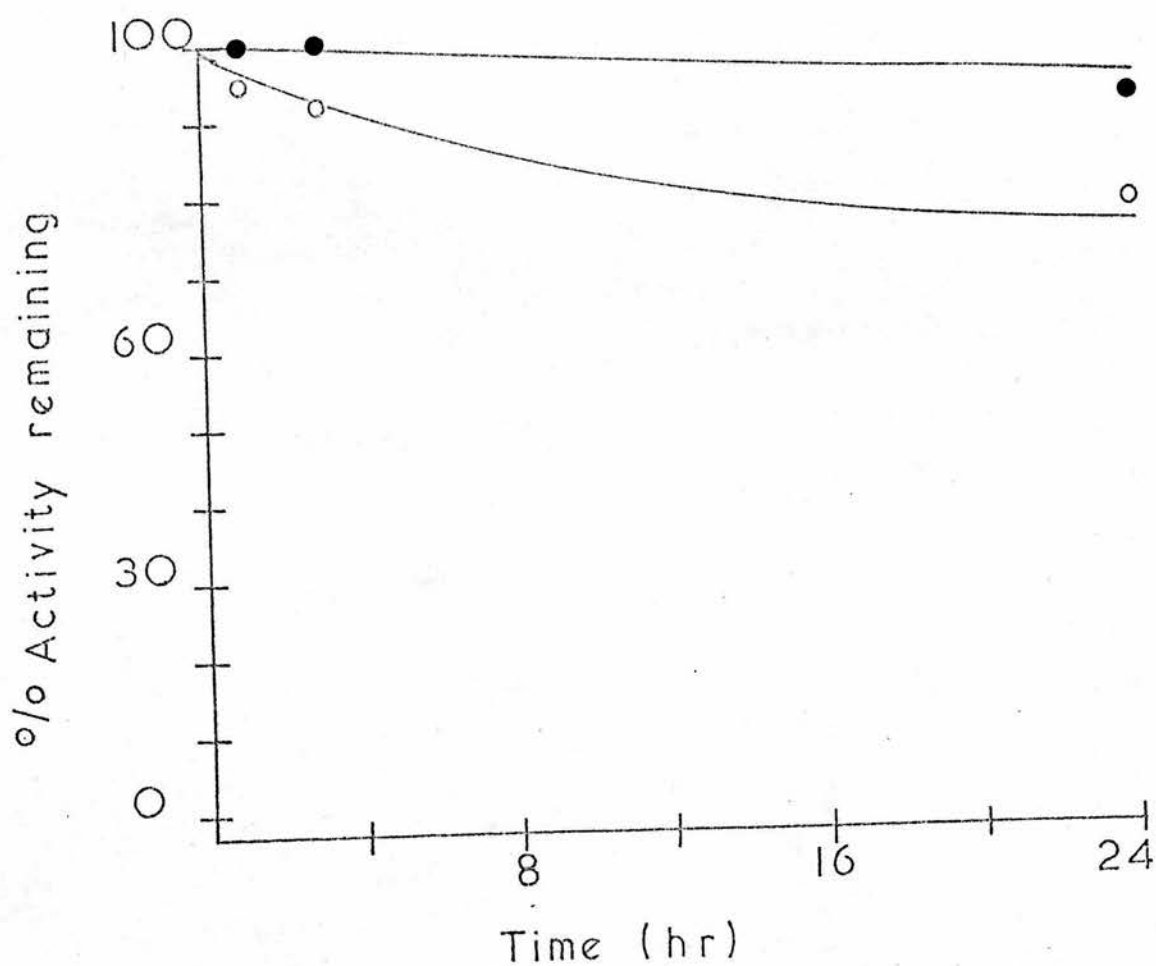


Fig. 5. Inactivation of pepsin C by BALOMe at pH 2.

- - control, without BALOMe
- - with BALOMe

extracts were dried over sodium sulphate and extracted with petroleum spirit (40-60°) to remove bromoacetic acid. The solvent was removed from the ethyl acetate layer on the rotary evaporator, leaving a yellow-brown oil. An attempt was made to crystallise this from acetone but crystal formation could not be induced.

The oil was subjected to the esterification procedure described for CALOMe but addition of ether failed to bring about any precipitation. The solvents were removed, the residual oil was extracted with ether and then dried in vacuo. Placing the oil in a vacuum desiccator brought about crystallisation but, on re-exposing the material to the air, it rapidly reverted to a sticky paste.

b) Attempted inhibition using crude BALOMe.

Incubations were set up as described for CALOMe but only at pH 2. 0.1 ml of a solution of 87 mg of the oil in 1 ml of methanol (i.e. a molar ratio of BALOMe/enzyme of 850/1) was added and the mixture was incubated at 20°. The results (Fig. 5) show that BALOMe was a very poor inhibitor since an 850 fold molar excess produced only 20% inhibition in 24 hours.

c) Criticism of CALOMe and BALOMe inactivations

It has been pointed out that it is highly likely that cyclisation of the chloroacetyl leucine and bromoacetyl leucine occurred under the alkaline conditions used for the syntheses so that CALOMe and BALOMe were never, in fact, synthesised. With the lack of elemental analyses for the compounds obtained, it is very doubtful whether the materials tested as inhibitors were genuine CALOMe and BALOMe so that it is not possible to make any deductions on the ability of these two compounds to inhibit pepsin C.

- a) sodium nitrite and acetic acid and
- b) amyl nitrite and acetic acid but a yellow oil was always obtained which could not be crystallised.

Dr. Stanford Moore (personal communication) indicated that he had had the same problem during the work on pepsin (Rajagopalan et al 1966b) and that this could be circumvented by using the racemic mixture of norleucine rather than the L optical isomer.

Glycyl-DL-norleucine could not be obtained commercially and so it had to be made in the lab.

6.4 Synthesis of diazoacetyl-DL-norleucine methyl ester (DNM) using chloroacetyl chloride.

The formation of the peptide bond was achieved by acylation of the norleucine under Schotten-Bauman conditions using chloroacetyl chloride. Amination of the chloroacetyl norleucine and subsequent esterification gave such a poor yield that this method of synthesis was discarded.

6.5 Synthesis of diazoacetyl-DL-norleucine methyl ester using N N' dicyclohexyl carbodiimide.

DL-norleucine methyl ester was synthesised using thionyl chloride in methanol. The yield was 95% of theoretical and the m.p. was 112° (lit. val. 115°).

Benzyloxycarbonyl glycine (Z-Gly) was prepared by dissolving 0.1 moles of glycine in 25 ml of 4N-NaOH in the cold and adding benzyloxycarbonyl chloride (0.11 moles) in five batches over a period of 40 minutes with stirring. Stirring was continued for 1 hour at 0° and then the alkaline solution was extracted three times with ether to remove



excess of reagent. The aqueous layer was acidified to pH 1 and left in the refrigerator overnight. The precipitate which had formed was filtered off and recrystallised from hot chloroform. The yield obtained was 6.7g (36%) and the m.p. was 120° (lit. val. 120°).

Coupling of the N-protected glycine with the norleucine ester was achieved using dicyclohexyl carbodiimide.

Since the hydrochloride salt of amino acid esters will not undergo this coupling reaction, the norleucine ester (0.03 moles) was suspended in 50 ml of ethylene dichloride and treated with an equimolar amount of triethylamine. This mixture was left to stand at room temperature for 2 hours, at which time the solid material present (presumed to be triethylamine hydrochloride) was filtered off, dried and weighed. The yield was 4.13g (theoretical yield 4.17g) and the m.p. was very high (over 250°).

To the solution of the free base of norleucine ester in ethylene dichloride, Z-glycine (0.032 moles) dissolved in 100 ml of a mixture of ethylene dichloride:tetrahydrofuran::1:1 at 0° , was added. Dicyclohexyl carbodiimide (0.0335 moles) in 3 ml of ethylene dichloride was added with stirring, giving molar ratios of norleucine ester/Z-glycine/carbodiimide::1:1.05:1.1.

Stirring was continued for 2 hours at 0° and then the mixture was left in the refrigerator overnight. A precipitate of dicyclohexyl urea appeared out of the yellow supernatant and this was filtered off and weighed. The

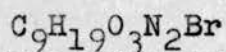
yield obtained did not make up the theoretical yield as dicyclohexyl urea is slightly soluble in ethylene dichloride.

The solvent was removed from the filtrate on the rotary evaporator and the yellow oil which remained was taken up in ethyl acetate. Petroleum spirit (40-60°) was added slowly and a yellow oil, with some white crystals mixed in it, appeared. The solvent was removed on the rotary evaporator and the oil was dissolved in a small volume of ethyl acetate. The solid material which remained was filtered off, dried and weighed and this made up the theoretical yield of dicyclohexyl urea (7.16g).

The filtrate was extracted with water, N-HCl, 0.25N-NaHCO₃ twice and the ethyl acetate layer was dried over sodium sulphate. This was filtered off and the solvent was removed to leave a yellow oil.

Glacial acetic acid (5 ml) and 15 ml of a solution of hydrogen bromide in acetic acid (4 fold excess) were added to the oil and the mixture was left to stand for 30 minutes at room temperature. Dry ether (500 ml) was added and the mixture was left to stand overnight. The supernatant was decanted off and the gummy material adhering to the bottom of the flask was dissolved in 5 ml of methanol. Dry ether was slowly added and crystallisation occurred. After standing overnight in the refrigerator the crystals were filtered off and once again re-crystallised from methanol and ether. The yield of glycyl-norleucine methyl ester was 5g (58%) and the m.p. was 119° (lit. val. 119°).

An elemental analysis was very kindly performed by Dr. J.W. Minnis, giving the following results:



Found: C 38.09; H 6.70; N 9.89; Br 27.97%

Theoretical: C 38.18; H 6.77; N 9.89; Br 28.21%

The dipeptide ester (684 mg) was diazotised by dissolving it in 2.5 ml of 2M sodium acetate and adding 320 mg of sodium nitrite and 0.125 ml of glacial acetic acid in the cold. The mixture was left for 2 hours in the refrigerator by which time it had solidified. The yellow solid was filtered off, washed with ice-cold water and dried in vacuo. The yield was 380 mg (74%) and the m.p. was 50° (lit. val. 50°).

This synthetic route gave a much better over-all recovery of material and all subsequent syntheses were done this way.

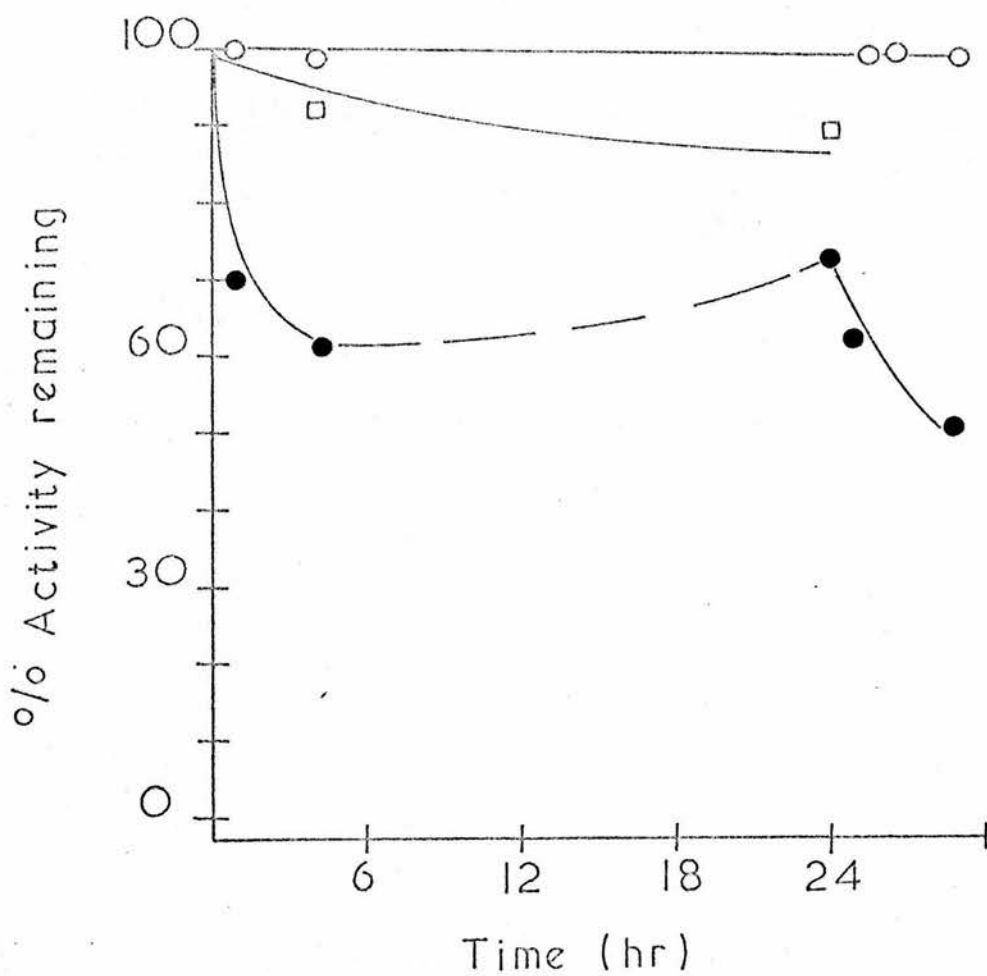


Fig. 6. Inactivation of pepsin C by a 900 fold excess of DNM at pH 2.

- - methanol plus cupric ions
- - DNM plus cupric ions
- - DNM

A second dose of inhibitor was added after 24 hours.

7. Inactivation of pepsin C by diazoacetyl
norleucine methyl ester (DNM).

These experiments to find the optimum conditions for inactivation of the enzyme were carried out in a final volume of 10 ml, at an enzyme concentration of 0.1-0.2 mg/ml, unless otherwise stated. The enzyme was dissolved in hydrochloric acid or acetate buffer of an appropriate pH and samples were taken for assay of haemoglobin digesting activity at various time intervals.

7.1 Attempted inactivation at pH 2.

Since the gastric proteases have a pH optimum around 2, if inactivation can be achieved at this pH, there can be no problems arising about the enzyme being in its most active configuration. It has been shown that cupric ions are required for activation of diazoketones (Zollinger 1961) and Rajagopalan has confirmed this in the inactivation of pepsin by DNM.

To 1 mg of enzyme in 10 ml of 0.01N-HCl was added 0.1 ml of 0.1M cupric acetate. This mixture was equilibrated to 20° and 6 mg of DNM in 0.1 ml of methanol was added (i.e. a final molar ratio of inhibitor/enzyme of 900/1). Controls were set up omitting a) the copper and b) the inhibitor, 0.1 ml of methanol being added instead. Incubation was continued at 20° and a great deal of effervescence was noted in the tube with all the reactants present. After 24 hours incubation an additional 0.1 ml of inhibitor solution was added. The results are shown

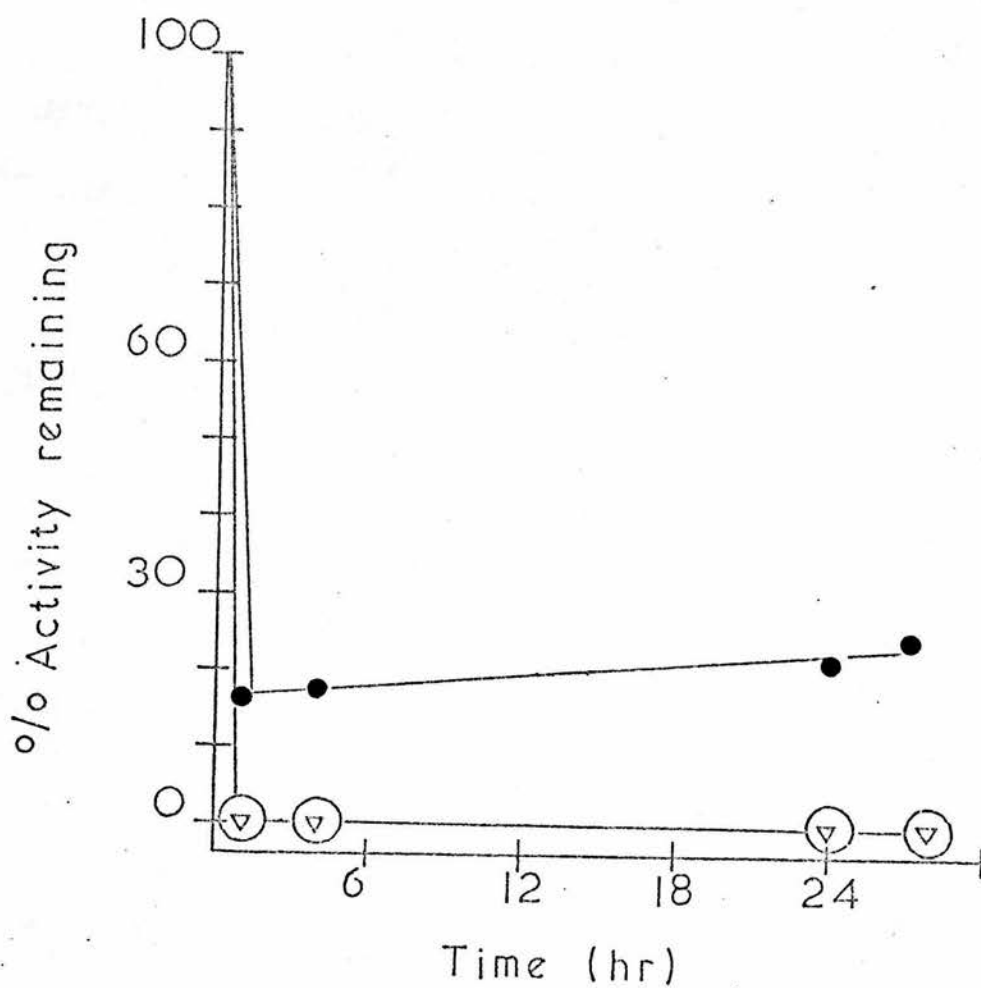


Fig. 7. Inactivation of pepsin C by a 900 fold excess of DNM at various pH values.

- - pH 4.0
- ▼ - pH 4.5
- - pH 5.6

in Fig. 6. The effervescence noted was presumably due to breakdown of the 'copper activated' inhibitor under the low pH conditions.

The control experiments show that pepsin C, at pH 2, was perfectly stable in the presence of a large excess of copper and in 1% methanol (cf. Tang 1966). Incubation of the enzyme with the inhibitor in the absence of copper produced a barely detectable loss in activity. Incubation in the presence of copper produced a 40% loss of activity in 4 hours and leaving the mixture for a further 24 hours appeared to produce a partial reactivation of about 10% but this may not be significant in view of later work. Addition of a further dose of inhibitor produced a subsequent drop in activity.

It would appear that at pH 2 the rate of decomposition of the inhibitor was too high to allow much interaction of the inhibitor with the enzyme.

7.2 Inactivation at various pH values.

Since the inhibitor appeared to be unstable at low pH values and pepsin C is irreversibly denatured above about pH 6, the inhibition attempts were made in the pH range 4-6.

Incubations were set up as before, using 0.2M acetate buffer, pH 4.0, 4.5 and 5.6, and after 25 hours the pH was lowered to 2.0. Fig. 7 shows the results. Above pH 4 a 900 fold excess of inhibitor completely inactivated the enzyme within 1 hour, whereas at pH 4 destruction of the inhibitor appeared to be still occurring to some extent and

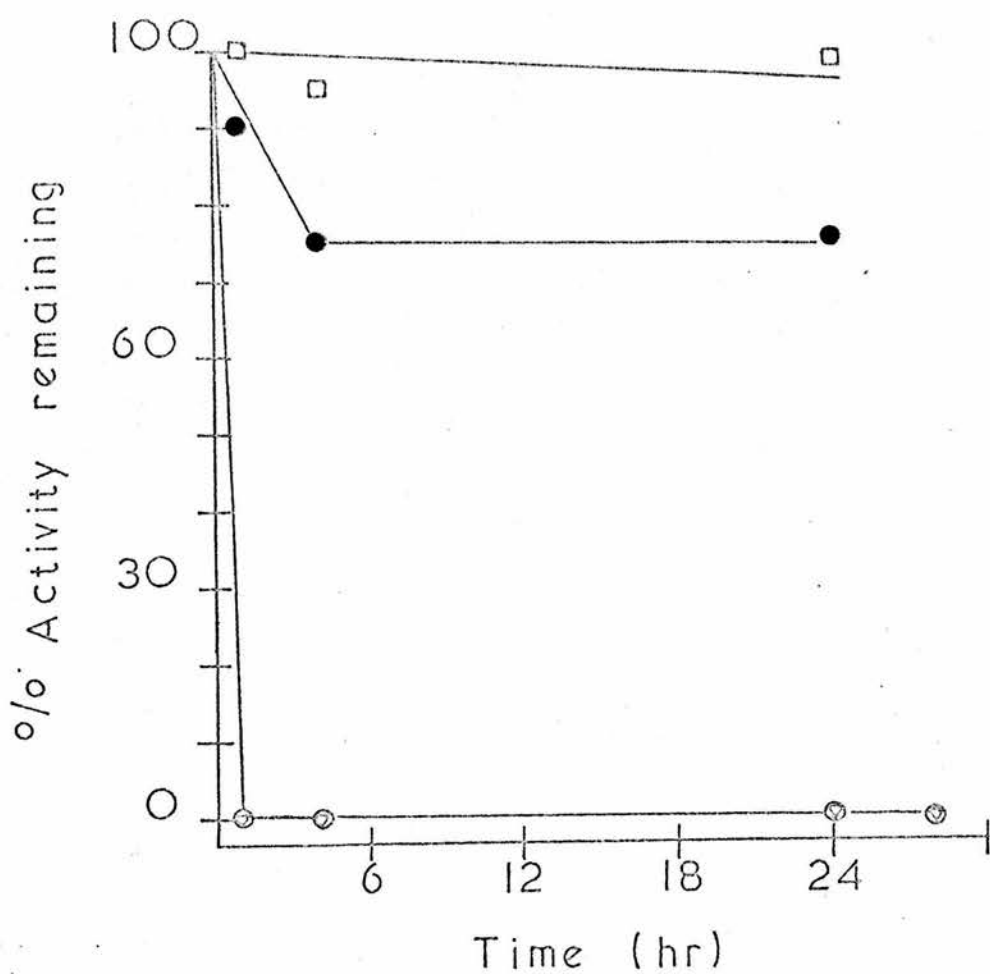


Fig. 8. Inactivation of pepsin C by DNM at small molar ratios of inhibitor/enzyme

Inhibitor/enzyme		
□	-	1/1
●	-	10/1
○	-	50/1
▼	-	100/1

the enzyme retained about 20% of its initial activity over a period of 24 hours. The enzyme-inhibitor complex once formed, however, appeared to be stable under acid conditions as shown by the fact that lowering the pH to 2 after 24 hours incubation and continuing the incubation at this pH produced no reactivation, even after another 24 hours.

7.3 Inactivation by smaller molar ratios of inhibitor/enzyme.

The excess of inhibitor/enzyme used previously was very high (900/1) and these experiments were designed to test whether smaller molar ratios would bring about inactivation.

Incubations were done at pH 4.6 in acetate buffer with addition of 0.1 ml of 0.1M copper acetate and the ratio of inhibitor to enzyme was varied from 1/1 to 100/1. (Fig. 8)

An equimolar mixture of DNM and pepsin C produced very little inhibition. A 10/1 molar ratio of inhibitor/enzyme produced about 25% inhibition in 4 hours and the activity remained at this level for a further 20 hours. Addition of a 50 or 100/1 molar ratio of DNM/enzyme brought about complete inactivation within 1 hour. The inhibited enzyme was not reactivated on standing for a further 20 hours or by lowering the pH to 2, which was done after 25 hours and incubating at this pH for another 24 hours.

7.4 Inactivation at various pH values at an inhibitor/enzyme ratio of 50/1.

As a 50/1 ratio of DNM/enzyme was able to produce complete inhibition within 1 hour at pH 4.6, the variation in activity over this period was measured at a variety of

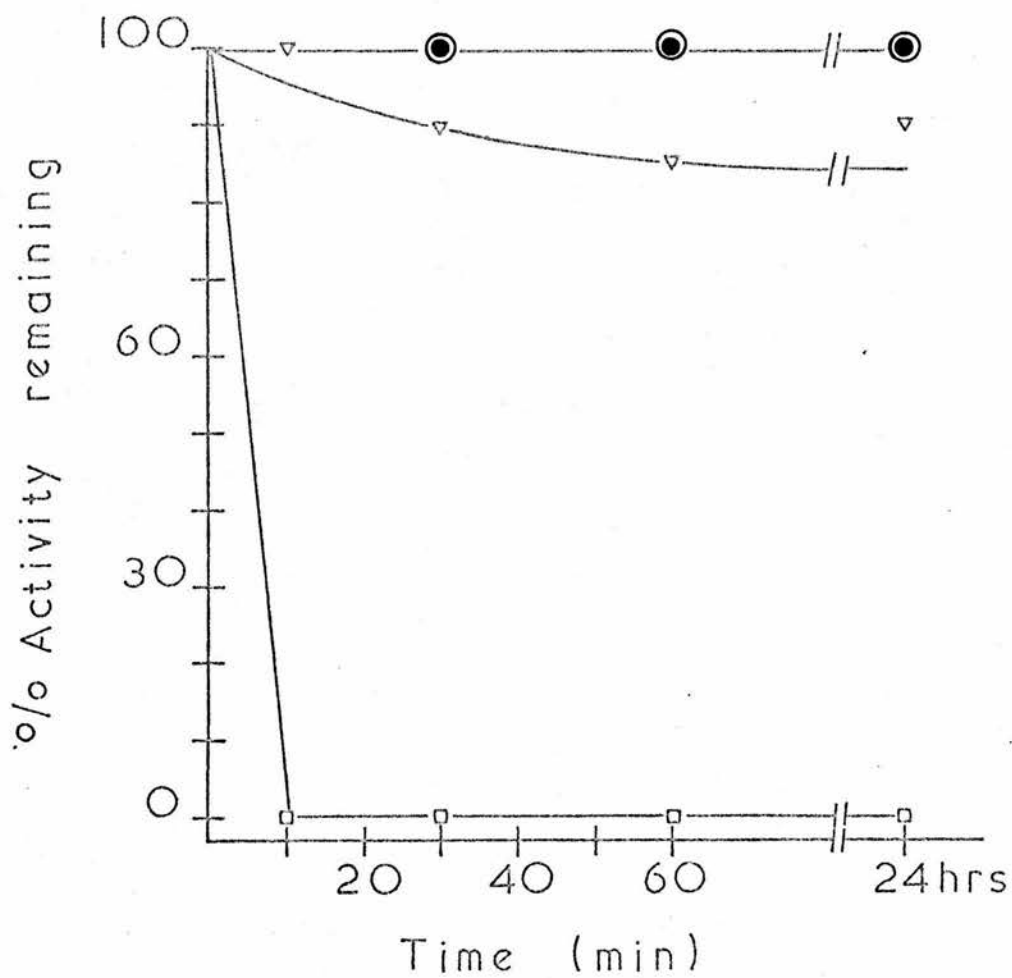


Fig. 9. Inactivation of pepsin C by a 50 fold excess of DNM at various pH values

	pH
● -	2.0
○ -	3.0
▼ -	4.0
□ -	5.0

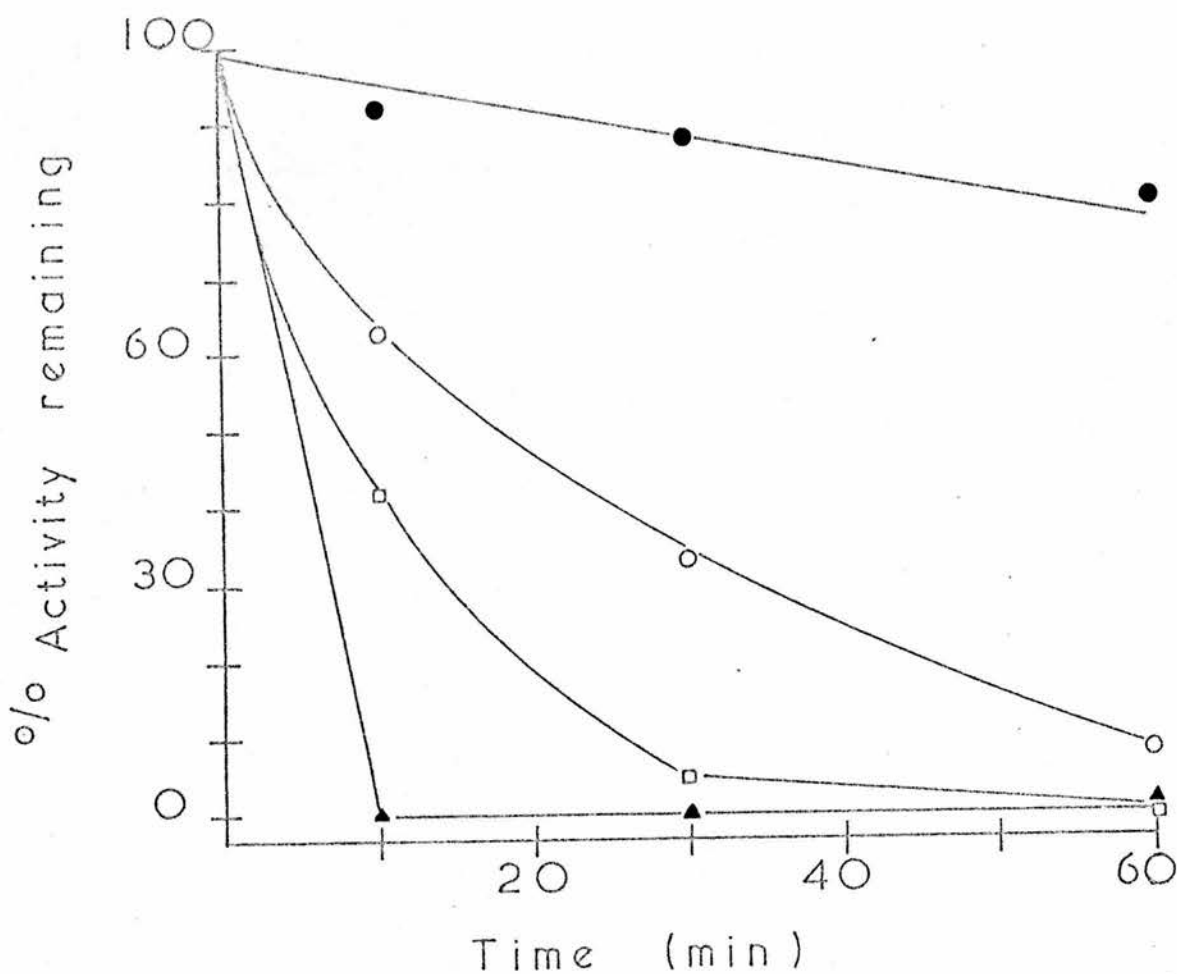


Fig. 10. Inactivation of pepsin C by a 40 fold excess of DNM at various molar ratios of Cu^{++} /enzyme

Cu^{++} /enzyme	
●	- 0/1
○	- 10/1
□	- 40/1
▲	- 80/1

pH values. Incubations were set up at 20° at pH 2, 3, 4, and 5, including 0.1 ml of 0.1M copper acetate and a 50 fold excess of inhibitor. The results are shown in Fig. 9.

As before, very little loss in activity was detected at pH 4 and below, but at pH 5 the enzyme was completely inactivated within 10 minutes and the inhibited enzyme was completely stable. At the lower pH values destruction of the inhibitor may occur under the acid conditions before the inhibitor can react with the enzyme.

7.5 Inactivation at various concentrations of cupric ions at an inhibitor/enzyme ratio of 40/1.

Since the experiments shortly to be described (Section 7.7) on the stoichiometry of the inactivation required rather more enzyme and in a more concentrated solution than the ones previously described, these incubations at varying cupric ion concentrations were done using 3.6 mg of enzyme in 3 ml of 0.2M acetate buffer, pH 5.0. This represents about a ten fold increase in the enzyme concentration.

The incubations were set up at 20° and molar ratios of cupric ions/enzyme from zero to 80/1 were used. The inhibitor was added in 40 fold excess and the results are shown in Fig. 10.

Inhibition in the absence of cupric ions was very slow, confirming that 'activation' of diazoketones into a reactive intermediate is carried out by cupric ions. An 80/1 excess of copper brought about a very rapid inactivation of the enzyme (within 10 minutes). As haemoglobin assays of

enzymic activity take about 30 minutes from the time of sampling to the reading of the extinction of the filtrate (Section 4.1) experimental procedure makes it difficult to take consecutive samples at a smaller time interval than about 10 minutes. Thus, it was judged that the very rapid inactivation brought about at an 80/1 molar ratio of cupric ions was too rapid to be followed properly.

7.6 Interpretation of the inactivation data.

These results can be rationalised fairly well, although there are one or two points which require further clarification.

Below pH 4, even with a vast excess of inhibitor, there is very little inhibition. At pH 4-4.5, a 900 fold excess of inhibitor produces 80% inhibition within 1 hour whereas, if the excess is cut to 50 fold, only 15% inhibition is achieved over the same period. This can be explained by assuming that at pH values between 4 and 4.5, there are two 'competing' reactions proceeding simultaneously. The first involves the inhibitor being destroyed by the acidity of the medium and the second is the interaction of the inhibitor with the enzyme. Thus, when a high molar ratio of inhibitor/enzyme is used, the destruction of the inhibitor can proceed but there is still sufficient inhibitor left to interact with the enzyme. However, at a molar ratio of inhibitor/enzyme of 50/1, the destruction of the inhibitor does not leave a high enough concentration of inhibitor to completely inactivate the enzyme.

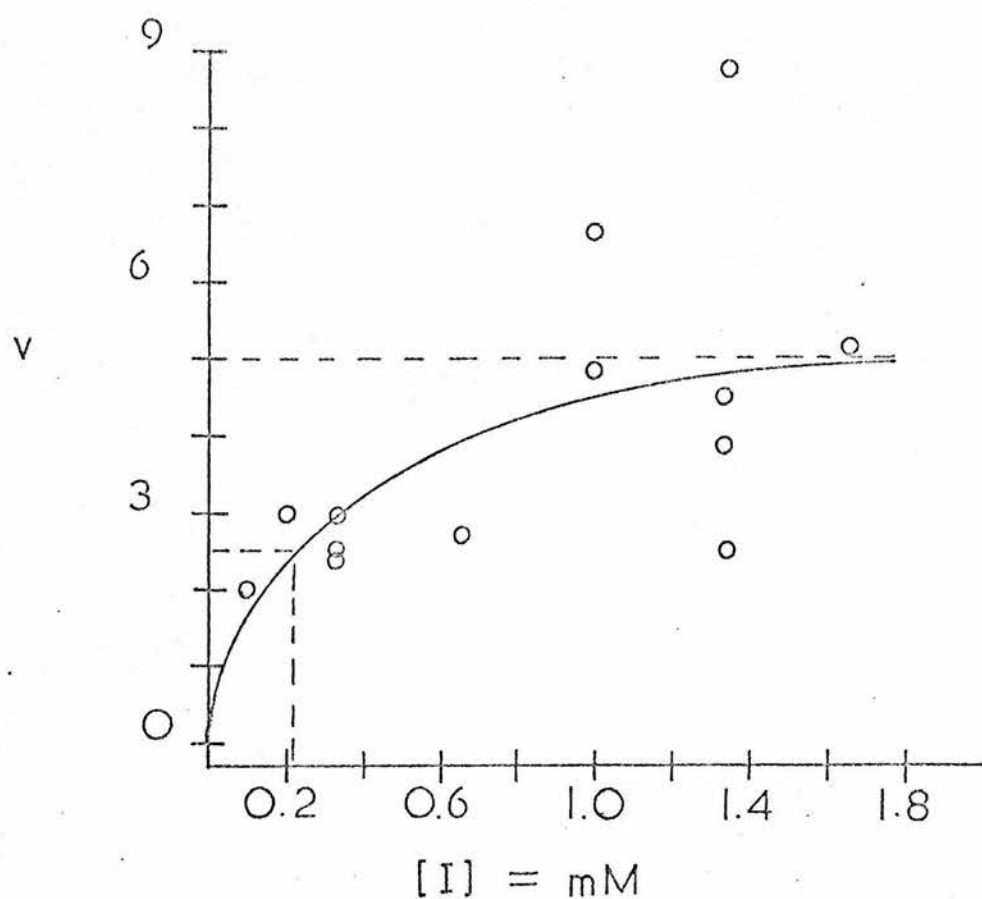


Fig. 11. Variation of the initial rate of inactivation of pepsin C by DNМ with inhibitor concentration.

From pH 4.5 to 5.5 the enzymic activity can be removed completely within 1 hour by using a 50 fold excess of DNM. However, if a smaller excess of DNM is used, there is a considerable variation in the loss of activity with time (which was put to good use in Section 7.7). Thus, in Fig. 8, a 10 fold excess of inhibitor produced only 25% inhibition in 4 hours whereas Fig. 15 shows that a 10 fold excess of DNM produced 45% inhibition within 1 hour.

This could be explained by any or all of the following:-

- a) Instability of the inhibitor,
- b) Different preparations of DNM were used,
- c) The inhibitor was not re-crystallised and can therefore only be termed 'crude'.

If, instead of looking at the over-all loss in activity with time, a plot of the initial rate of inactivation versus concentration of inhibitor is done at the same concentration of enzyme and cupric ions (Fig. 11) it appears that there is a saturation effect with the inhibitor, although it must be borne in mind that the values for the initial velocity are subject to a fairly large error because of the difficulties in carrying out haemoglobin assays within 10 minutes of each other. However, a very rough value for an apparent dissociation constant $K_M = 0.2 \text{ mM}$ can be found from this plot, the enzyme concentration being 0.033 mM . This must mean that DNM is bound fairly readily to a specific site and not that the inhibitor interacts with any group on the enzyme's surface. Since about a 50/1 molar ratio of DNM/enzyme has to be used at pH 5 to bring about total

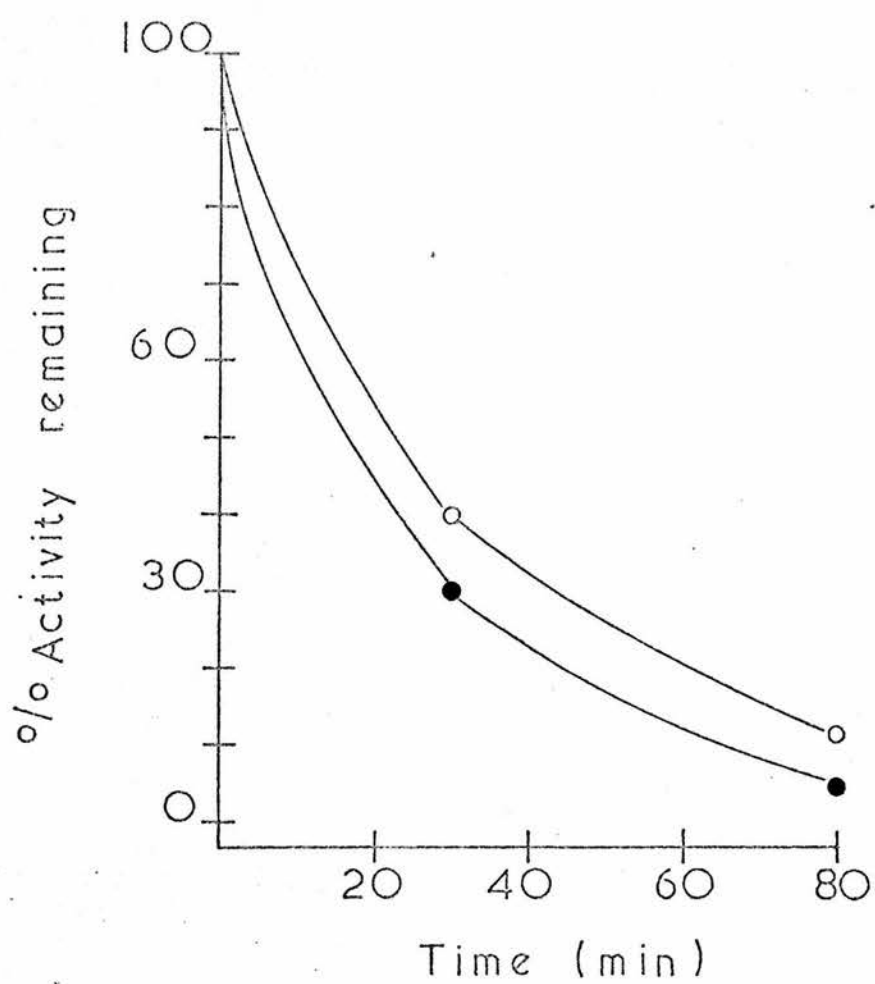


Fig. 12. Inactivation of pepsin C by DNM at different concentrations of enzyme.

[Enzyme] = mg/ml

○ - 0.3
● - 1.2

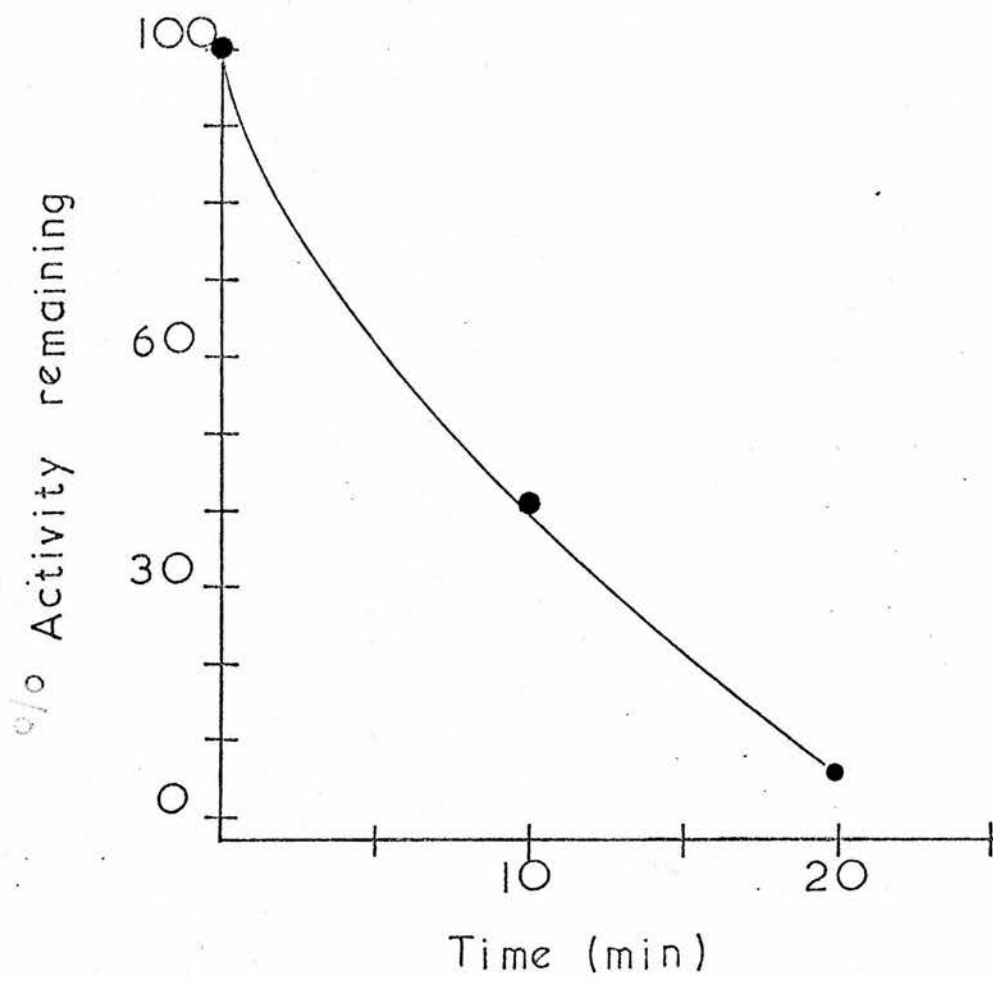


Fig. 13. Inactivation of pepsin C by DNM at an enzyme concentration of 2.0 mg/ml.

inactivation of the enzyme, this must reflect degradation of the inhibitor by reaction with the solvent before it can interact with the binding site on the enzyme.

A comparison was made of the inhibition of pepsin C at an enzyme concentration of 0.3 mg/ml and 1.2 mg/ml. A 100 fold excess of inhibitor was used and Fig. 12 shows that there was little difference in the rates of loss of activity. Thus, there seems to be no effect on the degree of inhibition on increasing the concentration of enzyme from 0.3 to 1.2 mg/ml. However, if the concentration of enzyme was increased beyond this to 2.0 mg/ml (the concentration used by Knowles in the analagous inhibition of pepsin) the inactivation under standard conditions proceeded in much the same way as at the lower concentrations (Fig. 13) but isolation and amino acid analysis of the inhibited enzyme revealed that a 1:1 incorporation of inhibitor had not been achieved since 1.8 residues of norleucine were found per mole of enzyme. This could be explained by an enzyme catalysed incorporation of a second residue of inhibitor into a second molecule of the E-I complex, which would be more important at higher concentrations of enzyme, since an $[E]^2$ or strictly an $[E][E-I]$ term would be involved.

As a result of these experiments, it was decided to use enzyme/cupric ions/DNM ratios of 1/30/40 at 20° and pH 5.0 and an enzyme concentration of 1.2 mg/ml. These conditions produced complete inactivation within 1 hour.

7.7 Stoichiometry of the inhibition of pepsin C by DNM.

This experiment was designed to find how the inactivation of the enzyme was related to the incorporation of the inhibitor into the enzyme. It was possible, by varying the molar ratio of inhibitor/enzyme from 1/1 to 50/1 (as previously discussed), to produce pepsin C inactivated to varying extents and this could be isolated (for determination of the inhibitor content) from the reaction mixture either by chromatography on Sephadex G-25 or by dialysis. The latter technique was used since it was more convenient for the preparation of a large batch of inhibited enzyme in a large volume for sequence studies (Section 8.2), although one large batch of inhibited enzyme (Section 14.1) was isolated by chromatography on Sephadex G-25.

In typical experiments, 3.6 mg of enzyme was dissolved in 3 ml of 0.2M acetate buffer, pH 5.0, and cupric acetate (0.03 ml of a 0.1M solution) was included. After equilibration to 20°, the inhibitor in 0.1 ml of methanol was added. After 1 hours incubation the mixture was either dialysed or chromatographed on a column of Sephadex G-25, (1 x 50 cm) equilibrated with water, collecting 2 ml fractions. A typical elution profile is shown in Fig. 14. Those fractions containing the protein were pooled, an equal volume of concentrated hydrochloric acid was added and the material was hydrolysed for 24 hours at 105° in vacuo. Amino acid analysis was done on the hydrolysate and the inhibitor content was found from the amount of norleucine present. The results obtained for Asp₂₈ Ile₁₄ and Arg₄ were

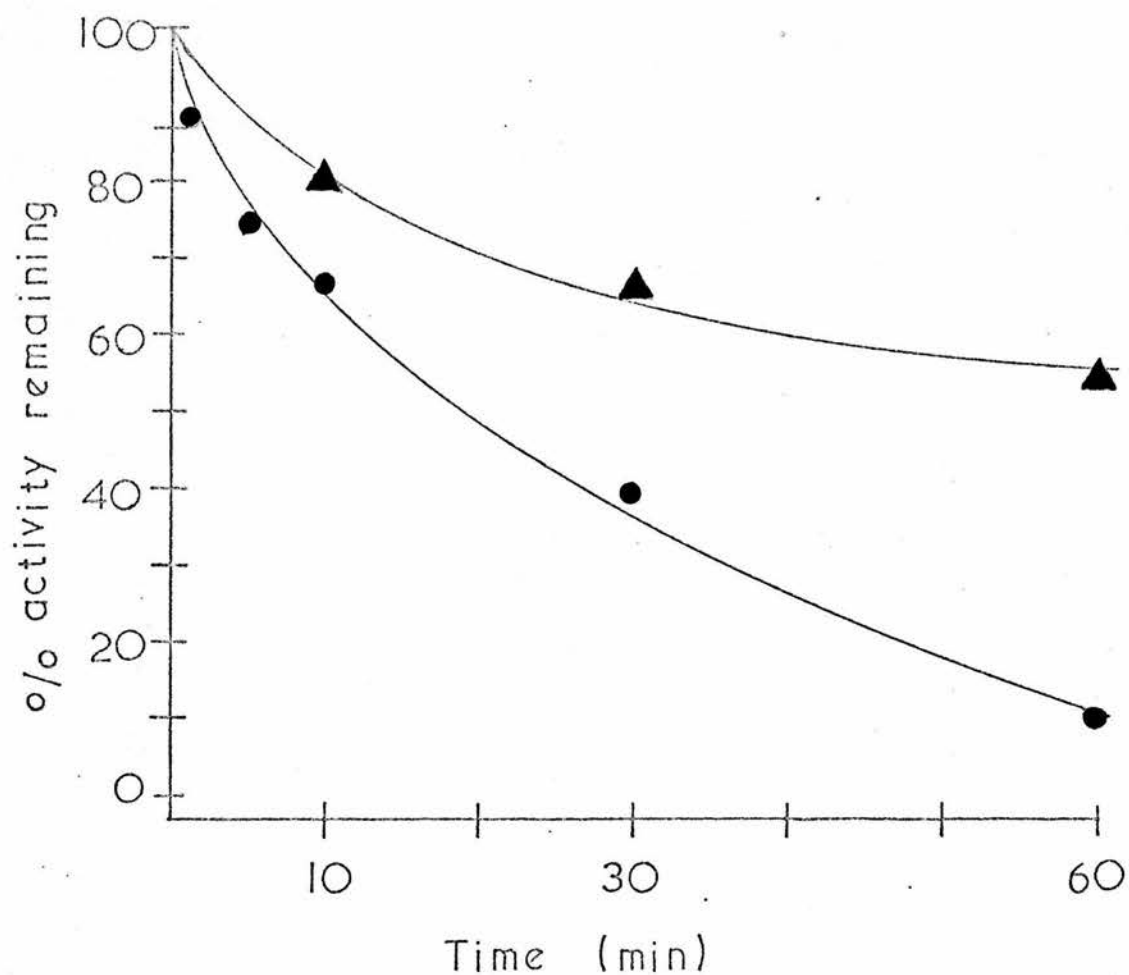


Fig. 15. Inactivation of pepsin C in the presence of a 30 fold excess of Cu^{++} at two different molar ratios of DNM/enzyme.

DNM/enzyme

▲ - 10/1

● - 40/1

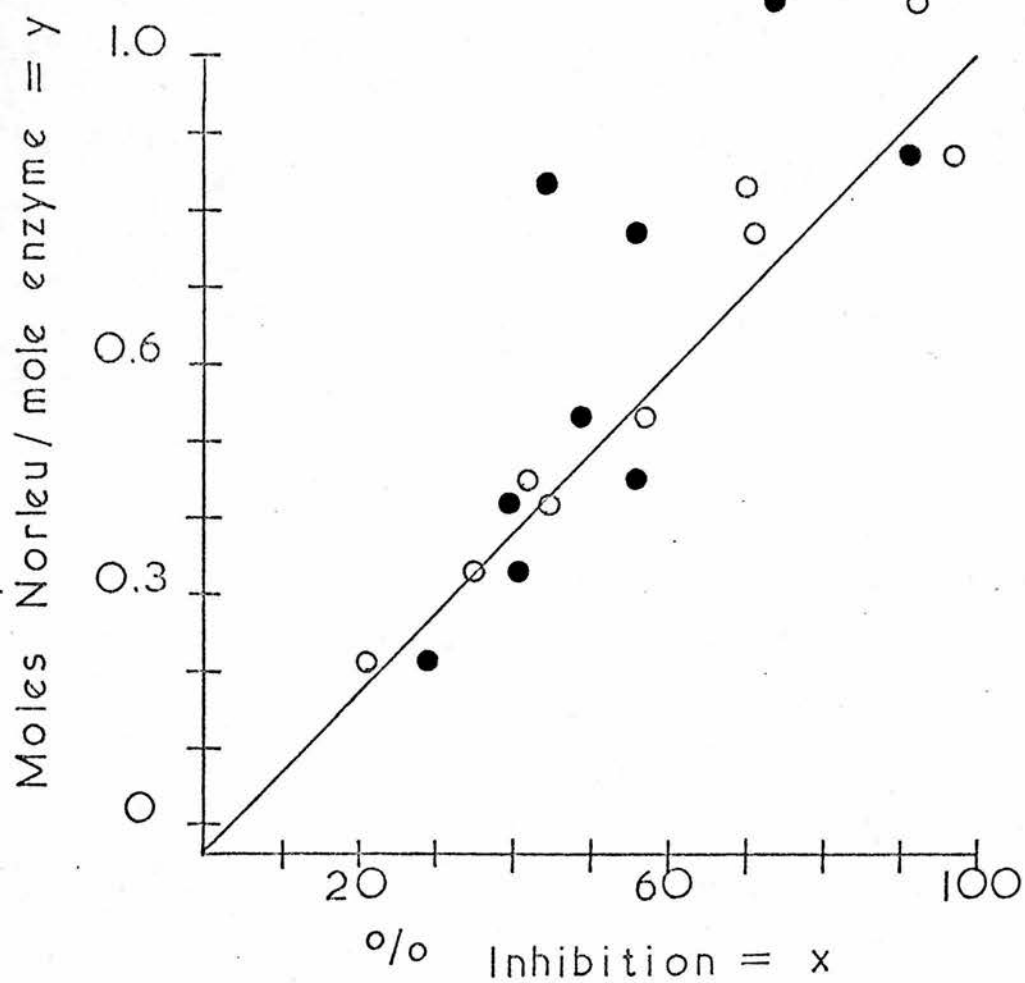


Fig. 16. Plot relating the degree of inhibition of pepsin C by DNM to the incorporation of inhibitor into the enzyme.

- - % inhibition after 60 min. incubation
- - % inhibition after dialysis or chromatography.

used to calculate the values for the other amino acids. Two typical inhibition profiles are shown in Fig. 15.

From the data collected from inhibition experiments such as these a graph can be plotted of moles of norleucine (i.e. inhibitor) incorporated versus percentage inhibition. This is shown in Fig. 16 with the degree of inhibition

a) after 60 minutes incubation (i.e. before dialysis or chromatography) and

b) after dialysis or chromatography both included.

In most cases very little additional inhibition occurred on dialysing the material and, since the non-diffusible material was hydrolysed and analysed immediately after assay, these figures for the activity are probably more accurate to use in calculating the degree of inhibition.

Fig. 16 shows that the theoretical straight line (100% inhibition \equiv 1.0 moles of Nle incorporated) fits these points.

Using the % inhibition values obtained after dialysis, the slope of a linear regression of y upon x, constrained to pass through the origin, is 0.0097 ± 0.0005 . A Student's t test shows that this does not differ from the theoretical 0.01 at the 5% level of significance. If no restriction is placed on the regression, a line is obtained whose slope is 0.0102 ± 0.0013 and whose intercept is -0.0004 ± 0.083 .

A variance ratio test shows that the addition of a term in x^2 to the regression does not improve the fit at the 5% level of significance. Thus the points lie just as well on the straight line as they do on a curve.

These results indicate that the inactivation of pepsin C by DNM proceeds with a concomitant incorporation of the inhibitor into the enzyme.

7.8 Significance of the inhibition.

It has been shown that DNM will inhibit pepsin C very specifically since only one residue of inhibitor is bound per mole of enzyme when the enzyme is completely inhibited. Due to the lack of a suitable peptide substrate it is not possible to say how DNM interacts with the enzyme. Thus, it cannot be said with any certainty that the inhibitor is acting at the active site of the enzyme but it certainly reacts very specifically with a particular group which must be involved in the catalytic activity of the enzyme, since interaction of this group with the inhibitor can produce a complete inactivation.

7.9 Protection of the inactivation by DNM by APD.

Ryle (unpublished observations 1970) has found that Ac-Tyr-Leu-Val-CySO₃H is a substrate for pepsin C with a K_M of 1.8 mM and k_{cat} of 208 min⁻¹. APD, a good substrate for pepsin, has been shown to be a competitive inhibitor of pepsin C at pH 2.0 with a K_I of 0.28 mM. One would expect that at pH 5.0 the rate of hydrolysis of Ac-Tyr-Leu-Val-CySO₃H by pepsin C would be too slow to allow any kinetic measurements to be made to show whether APD is also a competitive inhibitor at pH 5.0 but, if it can be shown that the rate of hydrolysis of Ac-Tyr-Leu-Val-CySO₃H is slowed down by the addition of APD, it is a fair assumption that APD inhibits competitively at pH 5.0.

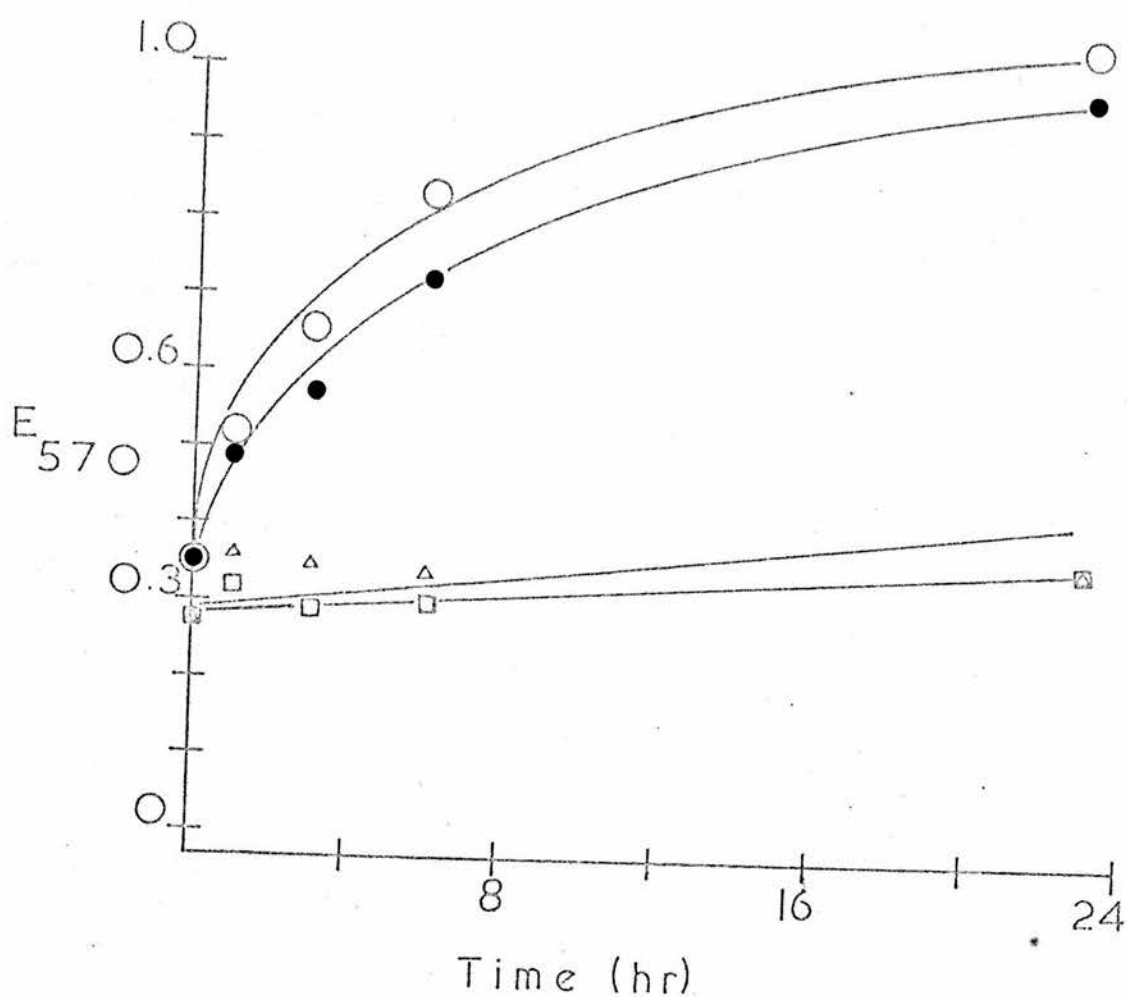


Fig. 17. Effect of APD on the hydrolysis of Ac-Tyr-Leu-Val-CySO₃H by pepsin C at pH 5.0.

- - Ac-Tyr-Leu-Val-CySO₃H
- - Ac-Tyr-Leu-Val-CySO₃H plus APD
- △ - APD
- - enzyme alone

Incubations were set up containing 12 ml of pepsin C solution (0.05 mg/ml) in 0.2M acetate buffer pH 5.0 and 0.25 mM in Ac-Tyr-Leu-Val-CySO₃H. APD was added to one so that the final concentration was 3 mM. Controls were included to account for a) autolysis of the enzyme and b) possible degradation of APD by pepsin C. Duplicate 1 ml samples were withdrawn at 0, 1, 3, 6 and 23 hours and added to 1 ml of a 2% ninhydrin solution (in 2 methoxyethanol:4M acetate buffer, pH 5.5, 3:1 v/v). These mixtures were boiled for 15 minutes in a water-bath, cooled, and 5 ml of a solution of 60% ethanol (v/v) was added. The extinctions were then read at 570 nm (Fig. 17).

The controls show that pepsin C did not degrade APD at pH 5, whereas it very slowly hydrolysed Ac-Tyr-Leu-Val-CySO₃H. In the presence of 3 mM APD the rate of hydrolysis was slowed down by about 10% so that the APD was inhibiting the hydrolysis of the substrate at pH 5.0. As predicted, the rate of hydrolysis of Ac-Tyr-Leu-Val-CySO₃H was too slow to allow any kinetic experiments.

Having shown that APD inhibits the action of pepsin C at pH 5.0 and since it is a competitive inhibitor at pH 2.0, if APD can be shown to protect the enzyme from inactivation by DNM this can be interpreted to mean that DNM binds at the active site of the enzyme. If, however, APD fails to protect against inhibition by DNM this does not necessarily mean that DNM does not react at the active centre, since this depends on the pseudo dissociation constants for E-DNM (0.2 mM - Section 7.6) and E-APD which is not known at pH 5.0.

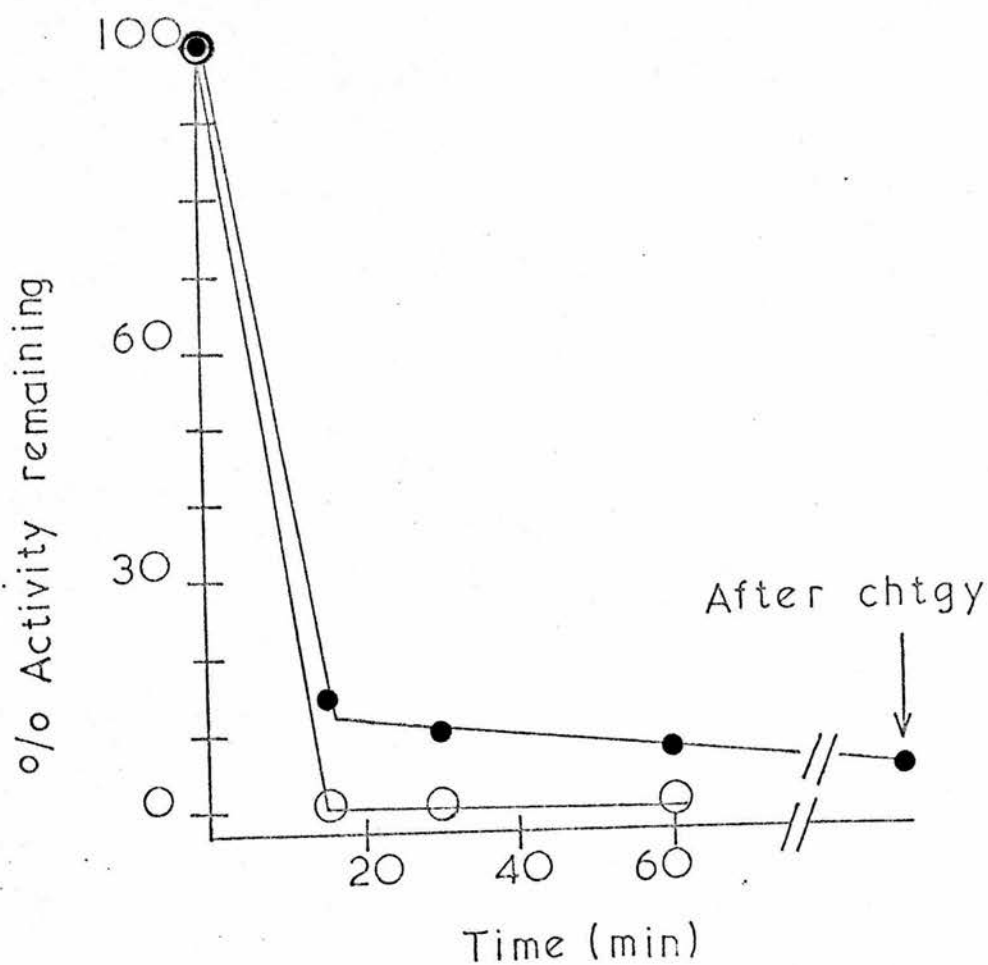


Fig. 18. Inactivation of pepsin C by a 40 fold excess of DNМ and the effect of APD on the inactivation.

- - minus APD
- - plus APD (3 mM)

Two incubations were set up containing 3.6 mg of pepsin C in 3 ml of acetate buffer, pH 5.0, and 0.03 ml of 0.1M cupric acetate was added to each. APD was added to one tube to give a final concentration of 3mM and, after equilibration to 20°, a 40 fold excess of DNM was added to each. Samples were taken for haemoglobin assay and after 60 minutes incubation the mixture containing the APD was chromatographed on a column of Sephadex G-25 (1 x 50 cm), equilibrated with water. The fractions containing the protein were pooled and after assay, hydrolysed and analysed. Fig. 18 shows the results of the incubations. The mixture containing APD retained about 10% more activity than the one without APD throughout the incubation. Thus, the APD appears to be protecting the enzyme from inactivation by DNM to an extent of 10%. Amino acid analysis of the protein-containing fractions after chromatography of the APD protected mixture showed an incorporation of 1.2 residues of norleucine/mole of enzyme and, since the enzyme was 95% inhibited, this still represents an incorporation fairly close to 1:1 in the presence of APD. It can also be shown from this experiment that APD does not interfere with the haemoglobin assays since the zero time assays of the protected and unprotected mixtures had exactly the same level of activity.

This presents some evidence, although by no means conclusive, that DNM interacts with pepsin C at the active site since it has been shown that APD is a competitive inhibitor and therefore must bind at the active site and this protects against inhibition by DNM to a small extent.

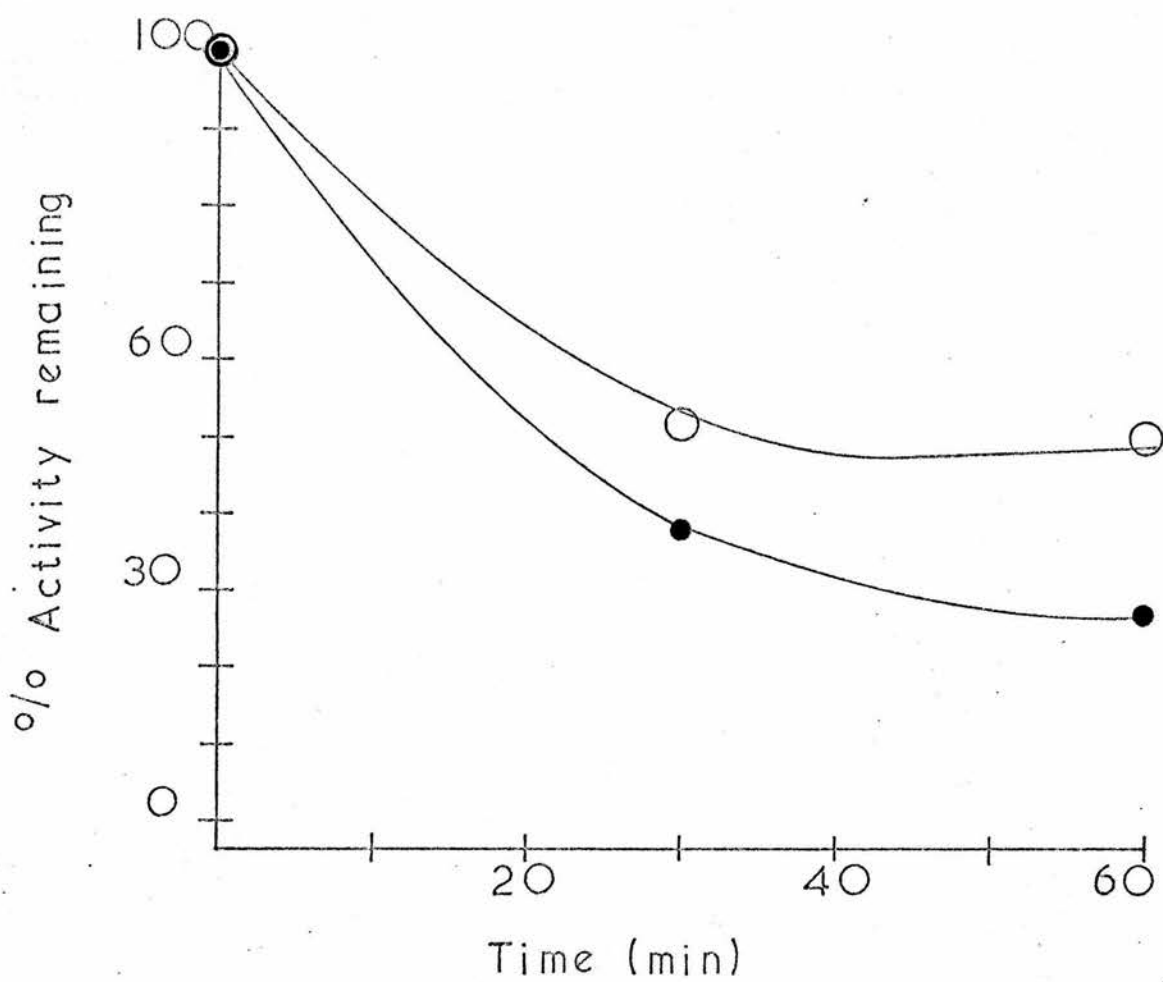


Fig. 19. Inactivation of pepsinogen C at pH 5.0 by DNM

Zymogen/Cu⁺⁺/DNM

- | | | |
|---|---|---------------|
| ○ | - | 1 / 30 / 40 |
| ● | - | 1 / 300 / 160 |

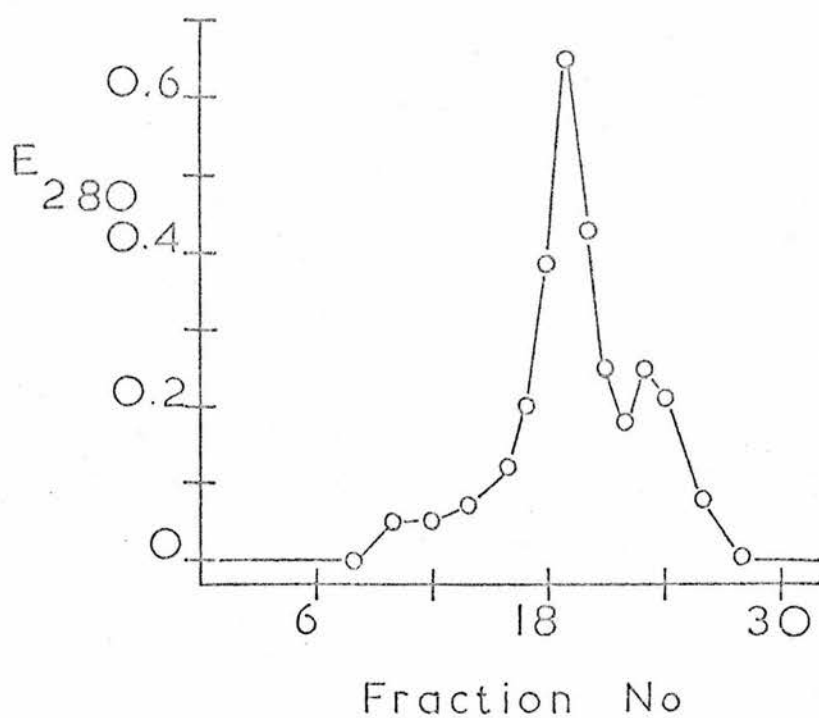


Fig. 20. Chromatography of incubation a) of zymogen and DNM on Sephadex G-25 (1 x 50 cm), equilibrated with water.

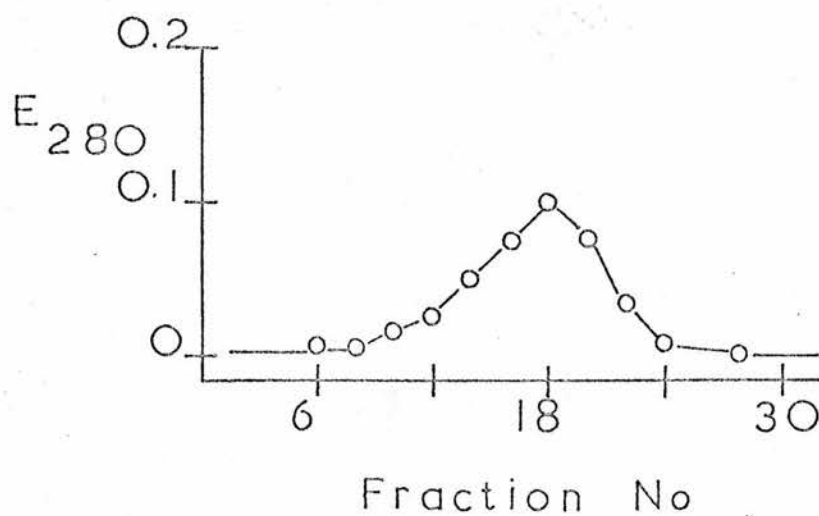


Fig. 20. Chromatography of incubation b) of zymogen and DNM on Sephadex G-25 (1 x 50 cm), equilibrated with water.

7.10 Experiments with pepsinogen C and DNM.

Rajagopalan et al (1966b) have demonstrated that pepsinogen only interacts with DNM to the extent of incorporating about 0.6 residues with a 280 fold excess of inhibitor and no incorporation occurs when the incubation is done at a 40 fold molar excess. Bayliss, Knowles & Wybrandt (1969) have shown that incubation of pepsinogen with diazoacetyl-L-phenylalanine methyl ester at pH 5.0 incorporates only 0.15 moles of inhibitor/mole of enzyme and if the incubation is done at pH 7 less than 0.04 residues of phenylalanine are incorporated. It appears from this that the binding site for diazoketones, present in pepsin, is not available to the inhibitor in the zymogen.

Similar experiments were tried with pepsinogen C. Zymogen (4 mg) was dissolved in 3 ml of acetate buffer, pH 5.0, and incubated with various concentrations of cupric ions and DNM. Fig. 19 shows the results of incubation with

a) a 30 fold excess of cupric ions and a 40 fold excess of inhibitor i.e. the standard conditions for incubation of the enzyme and

b) a 300 fold excess of cupric ions and a 160 fold excess of inhibitor.

After 60 minutes incubation the mixtures, which had become cloudy, were chromatographed on Sephadex G-25 (1 x 50 cm) with water as eluant. Fig. 20 shows the elution profiles.

The zymogen in these incubations had lost between 50 and 75% of its activity and the fact that the mixtures became cloudy indicated that something else had happened to the

	10-12	13-15	Pepsinogen C
Lys	6.8	—	12
His	1.0	—	2
Arg	4.0	—	7
Asp	40.5	31.9	30
Thr	20.5	21.1	25
Ser	31.0	30.5	35
Glu	63.5	48.9	46
Pro	11.9	15.1	20
Gly	33.2	32.0	35
Ala	21.1	20.4	23
Val	17.0	19.1	22
Met	—	1.4	5
Ile	13.7	14.2	16
Leu	24.9	32.7	40
Nle	1.4	2.7	—
Tyr	8.7	13.4	22
Phe	14.6	18.6	24

Table 2. Amino acid analyses of fractions obtained from chromatography of pepsinogen C incubation a) on Sephadex G-25.

protein. The elution profiles in Fig. 20 confirm that something had happened, either during the incubation or during the chromatography, since neither profile shows a protein peak in the expected position (cf. Fig. 14).

The fractions containing the absorbing material from incubation b) were pooled and hydrolysed and amino acid analysis revealed that there was more norleucine than any other amino acid. Since this material is chromatographing in the position expected for the excess of inhibitor, this is hardly surprising.

The fractions obtained from incubation a) were pooled as follows:-

10-12, corresponding to the normal elution volume of pepsin C,

and 13-15

These were hydrolysed and analysed. The amino acid compositions are shown in Table 2 along with the composition of pepsinogen C (Ryle & Hamilton 1966). Obviously these fractions obtained by chromatography of the incubation mixture did not contain pepsinogen C as such.

It was thought that the strange behaviour of the incubation mixtures during chromatography might be due to precipitation of the zymogen from solution at pH 5.0 which is near the isoelectric point of the zymogen. This would explain the cloudiness of the incubation mixtures and if solid zymogen was applied to the top of the column then it might be expected that slow re-dissolution of the protein would give the elution patterns observed.

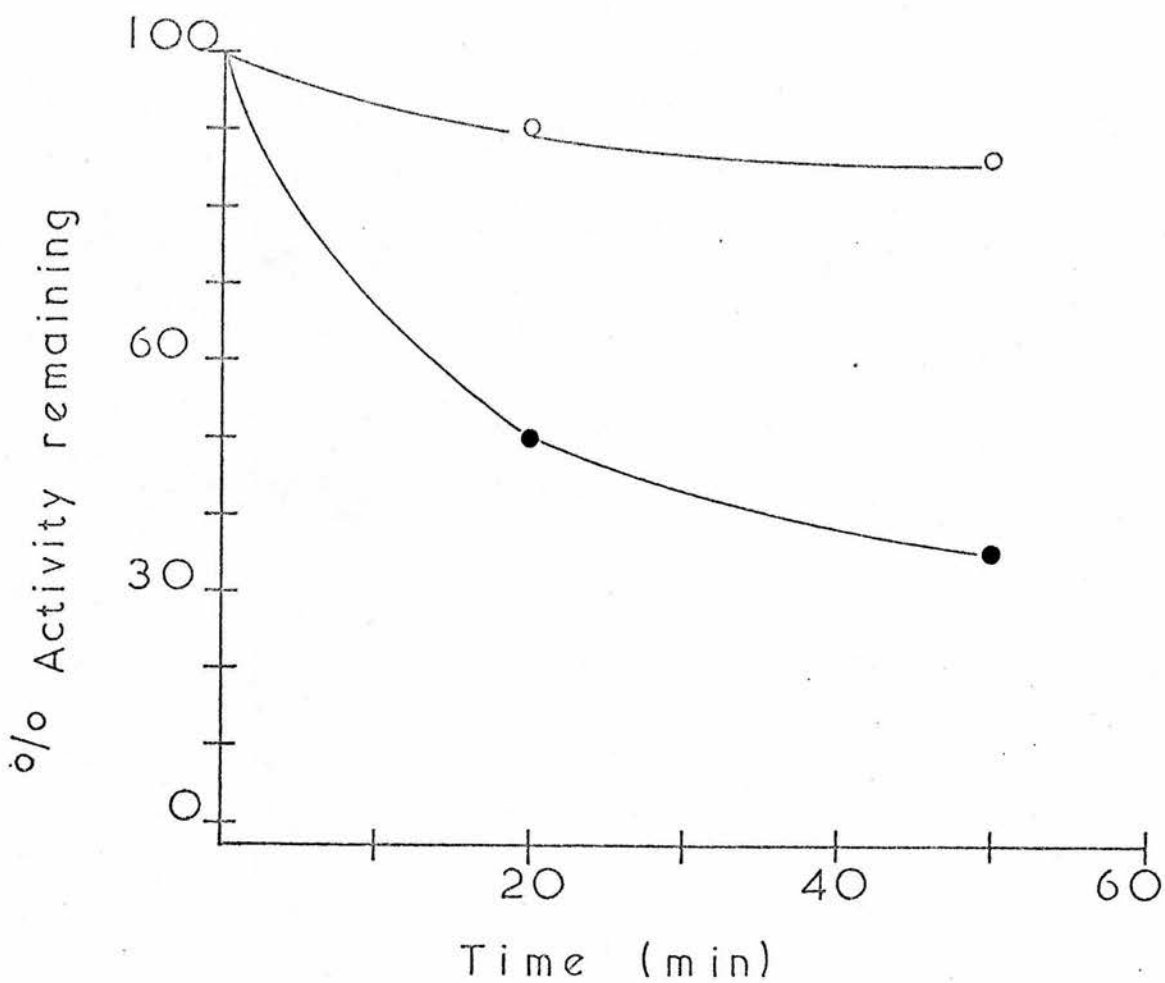


Fig. 21. Inactivation of pepsinogen C by a 40 fold excess of DNM at different pH values.

	pH
●	- 5.0
○	- 7.0

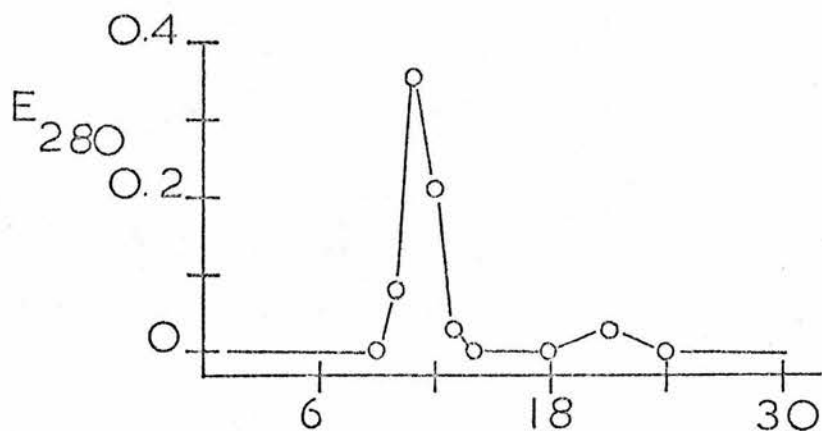


Fig. 22. Chromatography of the pH 5 incubation mixture of pepsinogen C and DNM on Sephadex G-25. (1 x 50 cm) equilibrated with 0.2M acetate buffer, pH 5.6.

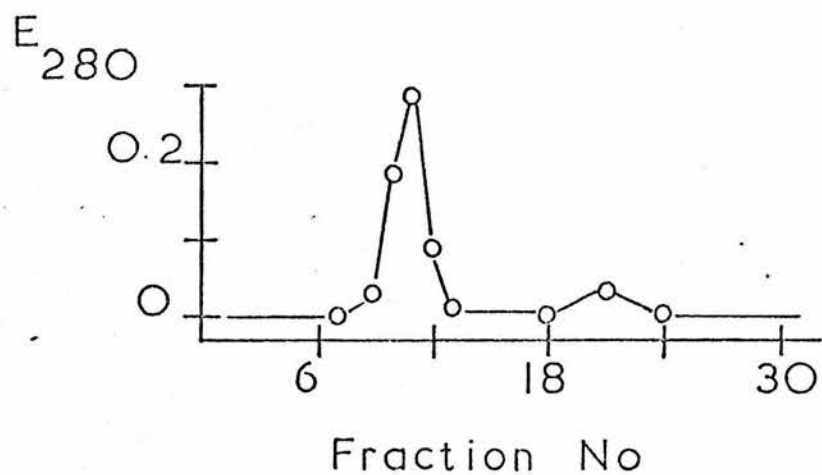


Fig. 23. Chromatography of the pH 7 incubation mixture of pepsinogen C and DNM on Sephadex G-25 (1 x 50 cm) equilibrated with water.

Since it was known that the zymogen was completely soluble at pH 5.6, another incubation was carried out with molar ratios of zymogen/cupric ions/DNM of 1/30/40 and at the end of incubation the pH was raised to 5.6 before the mixture was applied to the Sephadex column (equilibrated with acetate buffer, pH 5.6). The loss of activity with time is shown in Fig. 21, and Fig. 22 shows that the elution profile was 'normal' (cf. Fig. 14). After hydrolysis for 24 hours, amino acid analysis revealed the presence of 0.65 residues of norleucine per mole of protein, the zymogen having lost 65% of its potential activity.

A control was included to see whether the zymogen had undergone any activation to pepsin C at pH 5.0 i.e. the pH of the incubation. Pepsin C will clot milk at pH 5.6 whereas the zymogen will not and this assay can be used to follow the activation. The normal relative activity of pepsin C in $[P.U.]^{Ren}/_m[P.U.]^{Hb}$ is 0.21 and that of the zymogen is zero. It was found that, after incubation of pepsinogen C at pH 5.0 for 1 hour, the ratio was 0.01. Since both enzyme and zymogen have the same haemoglobin digesting activity, the zymogen being rapidly activated at the pH of the assay, this figure represents about a 5% activation of pepsinogen C to the active enzyme.

Another sample of zymogen (4 mg) was incubated at pH 7 in Tris-HCl buffer at molar ratios of protein/cupric ions/DNM of 1/30/40 and the rate of loss of activity is also included in Fig. 21. At the end of incubation the mixture was applied to Sephadex G-25, equilibrated with water,

and the elution profile, again 'normal', is shown in Fig. 23. The fractions containing the protein were pooled and after hydrolysis for 24 hours amino acid analysis showed that there were 0.1 moles of norleucine per mole of protein.

Incubation of DNM with pepsinogen C is thus rather complicated, but it seems that, in contrast to pepsinogen, pepsinogen C can bind DNM to varying extents with varying degrees of inhibition.

7.11 Experiments with alkali-inactivated pepsin C and DNM.

One other method of finding whether DNM reacted at the active site of the enzyme suggested itself. This was to look at the incorporation into denatured pepsin C which should have had sufficient structural disruptions to alter the configuration of the active site. Rajagopalan found with pepsin that 0.25 residues of inhibitor were incorporated into denatured pepsin.

This experiment was done with 3.6 mg of pepsin C in 3 ml of acetate buffer, pH 5.0. The pH of the solution was taken to 10 with alkali. The solution was left at room temperature for 30 minutes and then re-acidified to pH 5. A slight turbidity was noted on re-acidification. A 30 fold excess of cupric ions was added and after equilibration to 20°, in incubation

a) a 100 fold excess of DNM was added (¹⁴C-labelled inhibitor was used for this - Section 8.1) and in

b) a 40 fold excess of unlabelled inhibitor was added.

After 60 minutes the mixtures were dialysed against distilled

water. The radioactively-labelled inhibitor included in incubation a) was used to find whether any excess of inhibitor remained after dialysis for 24 hours with three changes of water (the standard conditions used).

The dialysed preparation (0.1 ml) was spotted onto Whatman No. 1 paper and a marker of 'Inhibitor Breakdown Products' - presumably hydroxyacetyl norleucine - (made by dissolving 4.7 mg of ^{14}C -DNM in a little methanol, adding 1 ml of 0.03N-NaOH and leaving this to stand overnight) was applied. The paper was subjected to electrophoresis at pH 3.6 at 2 kV for 1.5 hours and, after drying, scanned in a BTL 'Radiochromatogram Counter'. Fig. 24 shows that the dialysed preparation did have radioactivity in the region corresponding to the inhibitor breakdown products and a spot of radioactive material occurred at the origin. Staining with ninhydrin revealed a blue spot corresponding to this.

The material was re-dialysed until this test was negative. The protein was then hydrolysed and amino acid analysis showed that 0.5 residues of norleucine had been incorporated per mole of protein. This result may not be representative since the protein solution had been handled so much with opening the dialysis sac to test for the presence of inhibitor breakdown products and then re-dialysing the material.

Since removal of the excess of inhibitor had proved so difficult in this case, when incubation b) was carried out, the material was dialysed for 48 hours with five changes of water. The denatured enzyme precipitated in the sac and,

since unlabelled inhibitor had been used, it was not possible to check for complete removal of the excess of reagent. However, after hydrolysis, amino acid analysis showed that only 0.2 moles of norleucine had been incorporated per mole of protein and, since this figure was so low, it was assumed that dialysis had been effective.

DNM, then, does not interact with denatured pepsin C to any great extent, the level of incorporation being much the same as was found for pepsin and so the reaction which leads to the incorporation of the inhibitor seems to be fairly specific for active pepsin C.

As a result of the protection experiment and the experiments with denatured enzyme, it can be suggested that the group with which the inhibitor reacts occupies the active site, although the evidence is not as conclusive as it might be.

8. The site of attachment of DNM in pepsin C (I).

Having shown that DNM inactivated pepsin C very specifically, probably by reaction with an active site residue, it was obviously of interest to characterise the site of attachment by finding its position in the amino acid sequence around it. Isolation of the inhibitor containing peptide(s) would be facilitated if the inhibitor contained some easily detectable label.

8.1 Preparation of ^{14}C -diazoacetyl-DL-norleucine methyl ester.

Glycine- Cl^{14} (U) and glycine-1- Cl^{14} (0.5mCi of each) were diluted with 1g (13.3 mmoles) of unlabelled glycine and converted into glycyl-norleucine methyl ester by the synthetic route already described (Section 6.5). All side products, extraction solvents, sodium sulphate for drying etc. were assayed for radioactivity before being discarded. The yield of dipeptide ester obtained was 760 mg. Amino acid analysis on a hydrolysed sample of the material showed a Gly:Nle molar ratio of 1.06:1.00.

The dipeptide ester (500 mg) was subjected to diazotisation but a yellow oil formed and this could not be crystallised. A sample of the oil was incubated with 3.6 mg of pepsin C under standard conditions and the enzyme was found to be only 23% inhibited after 1 hour.

To check the purity of the glycyl-norleucine ester, a small sample of the radioactive dipeptide ester was subjected to electrophoresis at pH 2.0 with markers of glycine, norleucine ester and unlabelled glycyl-norleucine ester.

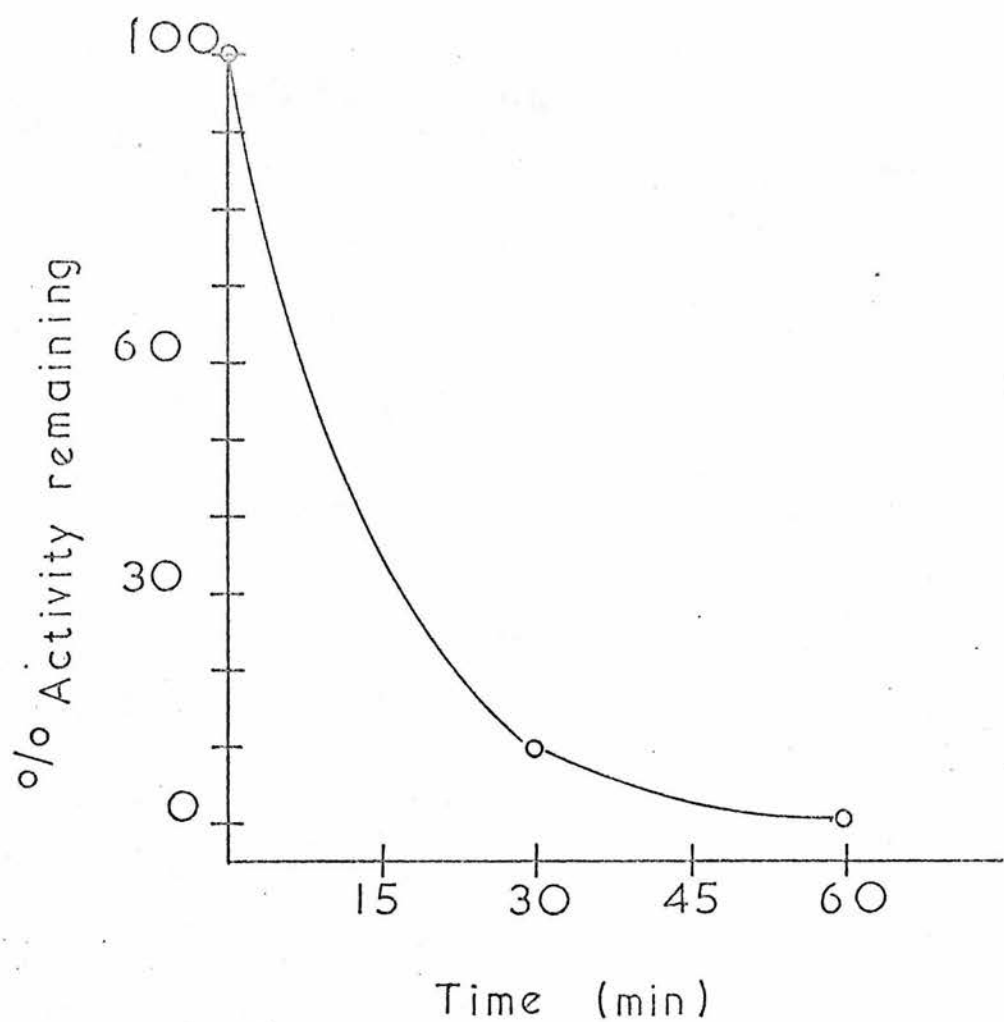


Fig. 25. Inactivation of a large batch of pepsin C by DNM.

The peptide migrated identically with the unlabelled dipeptide and did not contain any free glycine or norleucine ester.

The remaining 260 mg of radioactive dipeptide ester was diluted with 240 mg of unlabelled glycyl-norleucine ester and this mixture was diazotised. A seed crystal of DNM was added and this time the reaction mixture solidified. After 2 hours, the solid material was filtered off, washed with ice-cold water and dried in vacuo. The yield was 365 mg and the specific activity of the DNM was $37.5 \mu\text{Ci}/\text{mmole}$.

8.2 Preparation of a large batch of ^{14}C -labelled pepsin C.

Pepsin C (997 mg) was dissolved in 600 ml of 0.2M acetate buffer, pH 5.0 (1.66 mg/ml), contained in a conical flask and 0.1M cupric acetate (8.5 ml) was added. After equilibration to 20° , a solution of 350 mg of the solid DNM in 2 ml of methanol was added. This represents a molar ratio of inhibitor/enzyme of 40/1. The variation in activity with time is shown in Fig. 25. After 60 minutes the material was divided in two and both batches were dialysed separately for 70 hours with 15 changes of water. After opening the dialysis sacs, each batch was assayed separately for activity against haemoglobin and both were found to have 3% activity remaining. Both batches were tested by electrophoresis at pH 3.6, for the presence of inhibitor breakdown products (Section 7.11) and this was negative for both. Dialysis, then, had completely removed the excess of inhibitor so the protein was freeze-dried.

8.3 Calculation of the inhibitor content of inhibited pepsin C.

Pepsin C contains 28 aspartic acid residues per mole and amino acid analysis showed the inhibited enzyme to contain 2.89 moles of norleucine per 28 moles of aspartic acid. Determination of the inhibitor content of the inhibited enzyme from its specific radioactivity (2.3×10^{11} dpm/mole) and from that of the inhibitor (8.25×10^{10} dpm/mole) gave the value 2.79 moles of inhibitor per mole of inhibited enzyme.

The fact that a 1:1 incorporation was not achieved here may be related to the incubation having been done in a conical flask with a different surface area of glass/volume than in the pilot experiments done in test tubes or, more probably, because, in trying to minimise the volume of enzyme solution to be handled, the concentration of enzyme used was 1.7 mg/ml. This prompted the experiment mentioned in Section 7.6, to investigate the incorporation of the inhibitor into the enzyme at a concentration of 2 mg/ml. Since a 1:1 incorporation was not achieved there either, the explanation given before may be applicable to this large batch also.

However, this large batch of enzyme was too valuable to be discarded and it was thought that if the sites of attachment of these three inhibitors could be found it would be interesting to compare them with the site of attachment of a 1:1 labelled batch of enzyme to be prepared later.

9. Degradation of ^{14}C -inhibited pepsin C.

As a pilot experiment, 80 mg of inhibited pepsin C was digested with chymotrypsin and trypsin. Some considerable time was spent trying out various systems of column chromatography, electrophoresis and paper chromatography to see which would give the best method of isolating the labelled peptides. Perhaps not surprisingly, very little data on the composition of the labelled peptides was obtained from this.

To find which proteolytic enzyme would be most useful for the degradation, pilot digests on 10 mg of inhibited enzyme were done with the following enzymes, the hydrolysis products being subjected to electrophoresis at pH 3.6 for

I) 20 minutes at 300v

and then II) 90 minutes at 2 kV

a) Chymotrypsin and trypsin.

Inhibited pepsin C (10 mg) was dissolved in 2.5 ml of 0.2M ammonium bicarbonate and 0.1 ml of a solution of 1.9 mg of chymotrypsin in 1 ml of 0.001N-HCl was added to give a final molar ratio of enzyme/substrate of 1/30. After 3 hours at 37°, 0.1 ml of a solution of trypsin (1.7 mg in 2 ml) was added and the incubation was continued for a further 3 hours. The digestion mixture was dried down in vacuo and then 0.2 ml of 5% acetic acid was added. 0.075 ml of this was taken for electrophoresis.

b) Thermolysin.

Inhibited pepsin C was dissolved in 2.5 ml of 0.2M ammonium bicarbonate containing 0.003M calcium ions and

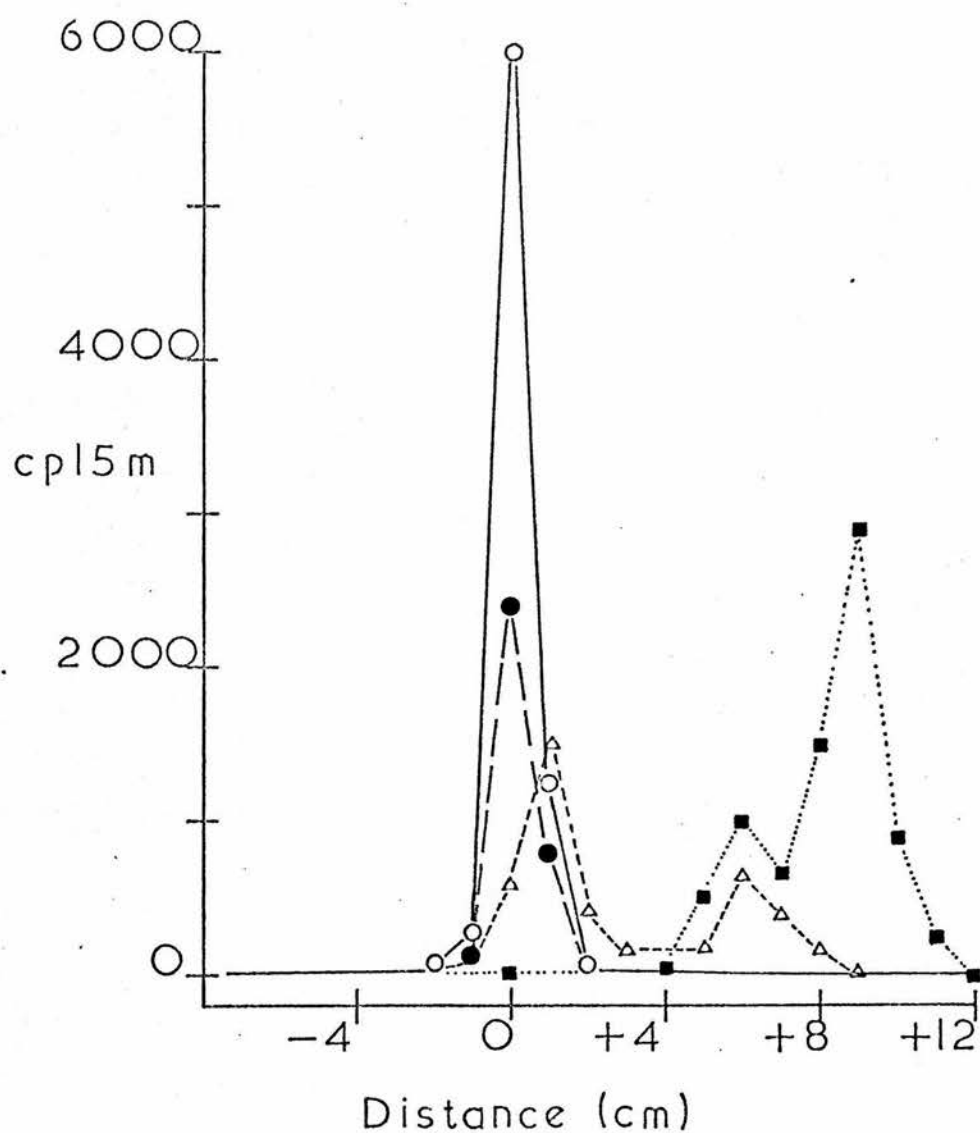


Fig. 26. Electrophoresis at pH 3.6 of pilot digests of inhibited pepsin C.

- △--- - Chymotrypsin and trypsin
- - Thermolysin
- - Pepsin
-■..... - Inhibitor breakdown products

0.1 ml of a solution of 1.7 mg of thermolysin dissolved in 2 ml of ammonium bicarbonate was added. After 6 hours at 37° the digestion mixture was dried down in vacuo. 5% acetic acid (0.1 ml) was added and 0.025 ml was taken for electrophoresis.

c) Pepsin.

Inhibited pepsin C was suspended in 2.5 ml of 0.02N-HCl and 0.1 ml of a solution of 2 mg of pepsin in 1 ml of water was added. After 6 hours at 37° some insoluble material was present and this was centrifuged off and assayed for radioactivity. This contained only 10% of the total radioactivity. The supernatant was dried down in vacuo and re-dissolved in 0.1 ml of 5% acetic acid. 0.05 ml was taken for electrophoresis.

Fig. 26 shows the patterns obtained from electrophoresis at pH 3.6 of these three digests with inhibitor breakdown products (Section 7.11) for comparison. Staining the strips with ninhydrin revealed a large amount of material clustered round the origin with a few peptides having migrated. It was thought that the papers might have been overloaded so that the peptides were producing a local high salt concentration effect. As a result, more dilute solutions of the thermolysin and pepsin digests were subjected to electrophoresis at pH 6.5 at 2 kV for 90 minutes. The papers had now to be scanned for much longer and 16 hours was found to be a convenient period. To show whether the counts obtained differed significantly from background, a Student's t test was done (Moore & Edwards 1965) using the

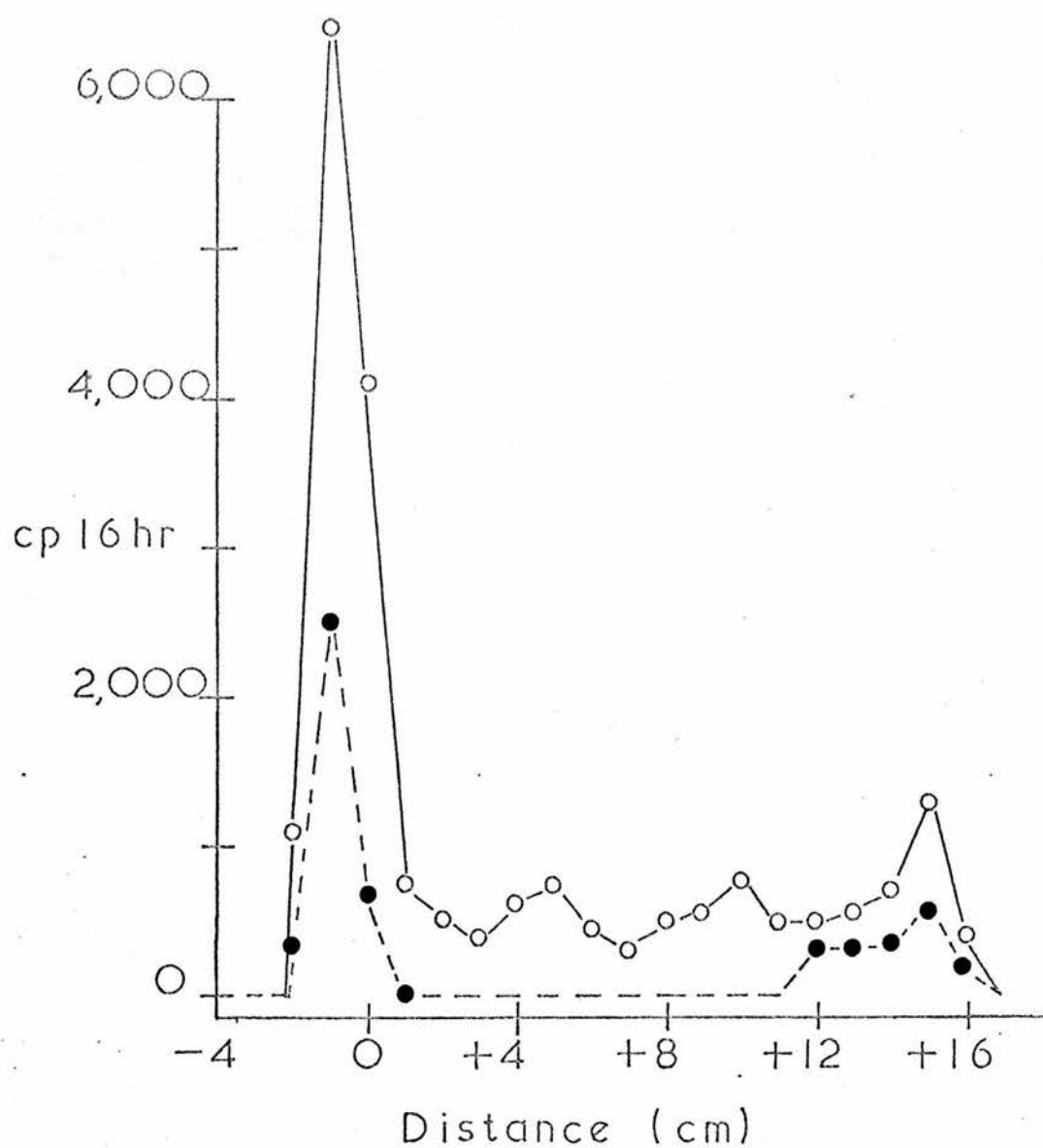


Fig. 27. Electrophoresis at pH 6.5 of "diluted" digests.

-●- Thermolysin
 -○- Pepsin

formula:-

$$t = \frac{x_1 - x_2}{s \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \quad \text{where} \quad s^2 = \frac{(n_1-1)s_1^2 + (n_2-1)s_2^2}{n_1 + n_2 - 2}$$

and x_1 = number of counts from sample, counted n_1 times,
and x_2 = counts from background, counted n_2 times.

Fig. 27 shows the scans obtained from this electrophoresis and it was found by applying the above test that all the peaks were significant to a level of at least 98%.

d) Papain.

Inhibited pepsin C (12 mg) was dissolved in 1 ml of a solution 5 mM with respect to cysteine containing 1 mM EDTA (Smyth 1967) and the pH was adjusted to 7 with alkali. 0.1 ml of a solution of papain (6 mg/10 ml) was added and this mixture was incubated at 37° for 6 hours. Some insoluble material had appeared by the end of the incubation and this was centrifuged off, washed with water, re-sedimented and the washings were added to the supernatant. Assay of the precipitate for radioactivity showed it to contain about 20% of the total. After drying the supernatant in vacuo, 0.5 ml of 5% acetic acid was added. The material was not soluble in this but addition of a few drops of alkali readily solubilised it. Since the material was not soluble in 5% acetic acid electrophoresis at pH 3.6 was pointless.

0.01 ml of the solution was used for electrophoresis at pH 6.5.

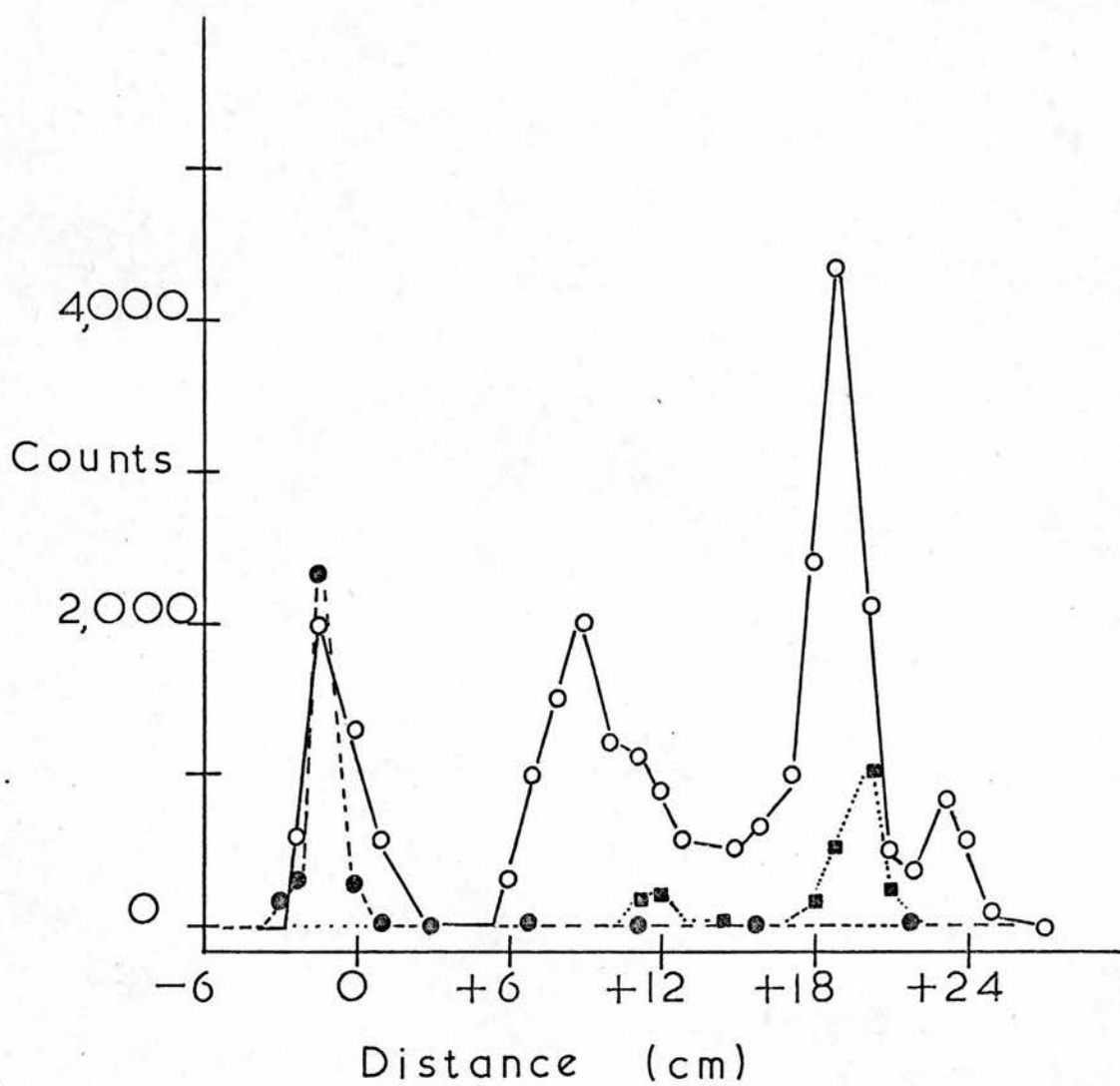


Fig. 28. Electrophoresis at pH 6.5 of pilot digests of inhibited pepsin C.

- - Papain (cp4hr)
- - Subtilisin (cp16hr)
-■..... - Inhibitor breakdown products (cp5m)

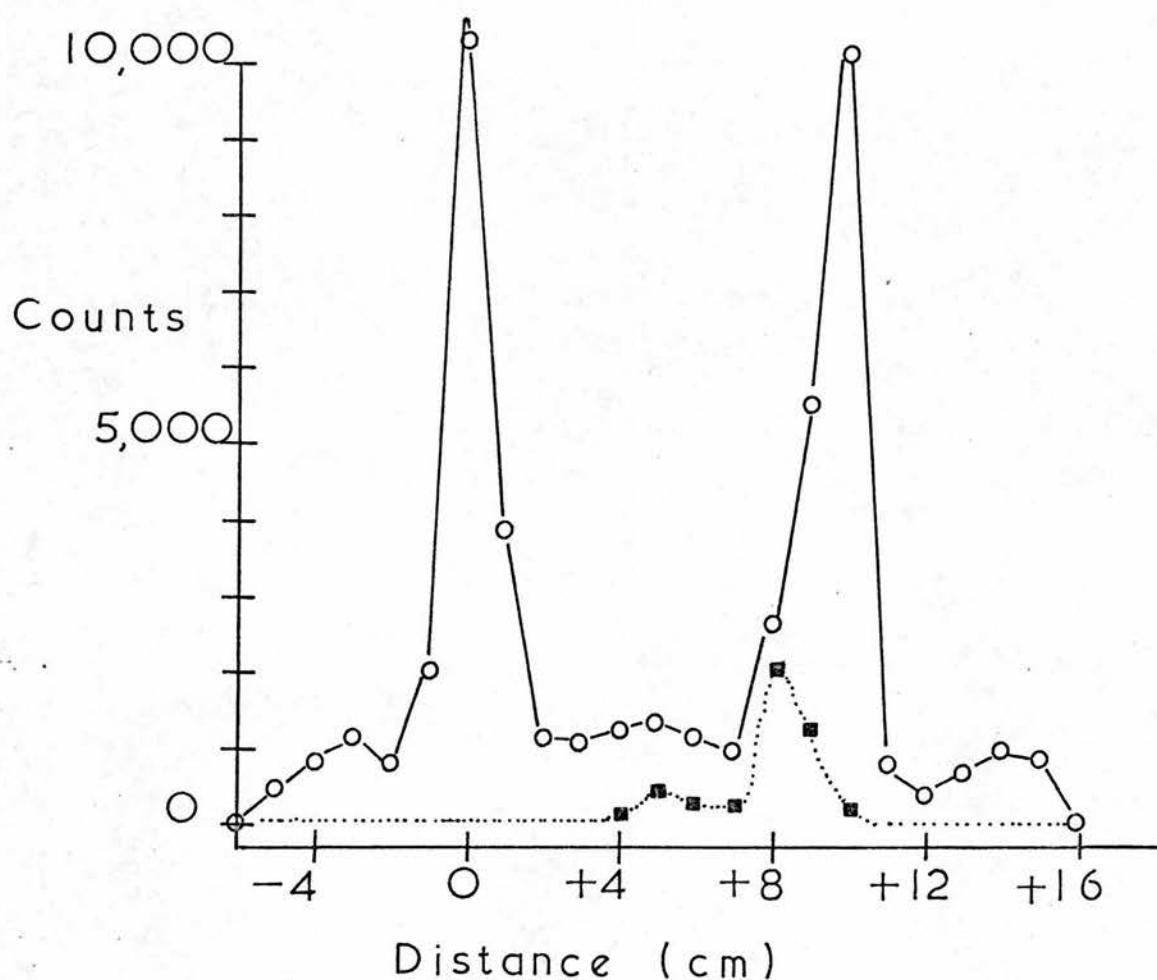


Fig. 29. Electrophoresis at pH 3.6 of pilot subtilisin digest.

—○— - Subtilisin (cp16hr)
■..... - Inhibitor breakdown products (cp5m)

e) Subtilisin.

Inhibited pepsin C was dissolved in 1 ml of 0.1M ammonium bicarbonate and 0.1 ml of a solution of subtilisin (1 mg/ml) was added. This was incubated at 37° for 6 hours. The material was dried down in vacuo and dissolved in 0.5 ml of 5% acetic acid.

The acetic acid solution (0.01 ml) was applied to No.1 paper and run at pH 6.5 at 2 kV for 90 minutes along with inhibitor breakdown products.

Fig. 28 shows the scans obtained from the papain and subtilisin digests. Application of the t test showed that the peaks were significant to a level of > 99.9%.

Electrophoresis at pH 3.6 of 0.01 ml of the subtilisin digest gave the pattern shown in Fig. 29. The fastest travelling band from the subtilisin digest migrated consistently slightly faster than the inhibitor breakdown products at pH 3.6 and a little slower at pH 6.5.

As a result of these digests it was decided that either pepsin or subtilisin would be the most useful enzymic degradation to employ since digestion with these gave rise to more than one labelled band on electrophoresis. Subtilisin was used in the hope that small peptides could be isolated initially and these could be expanded later by using a different method of digestion.

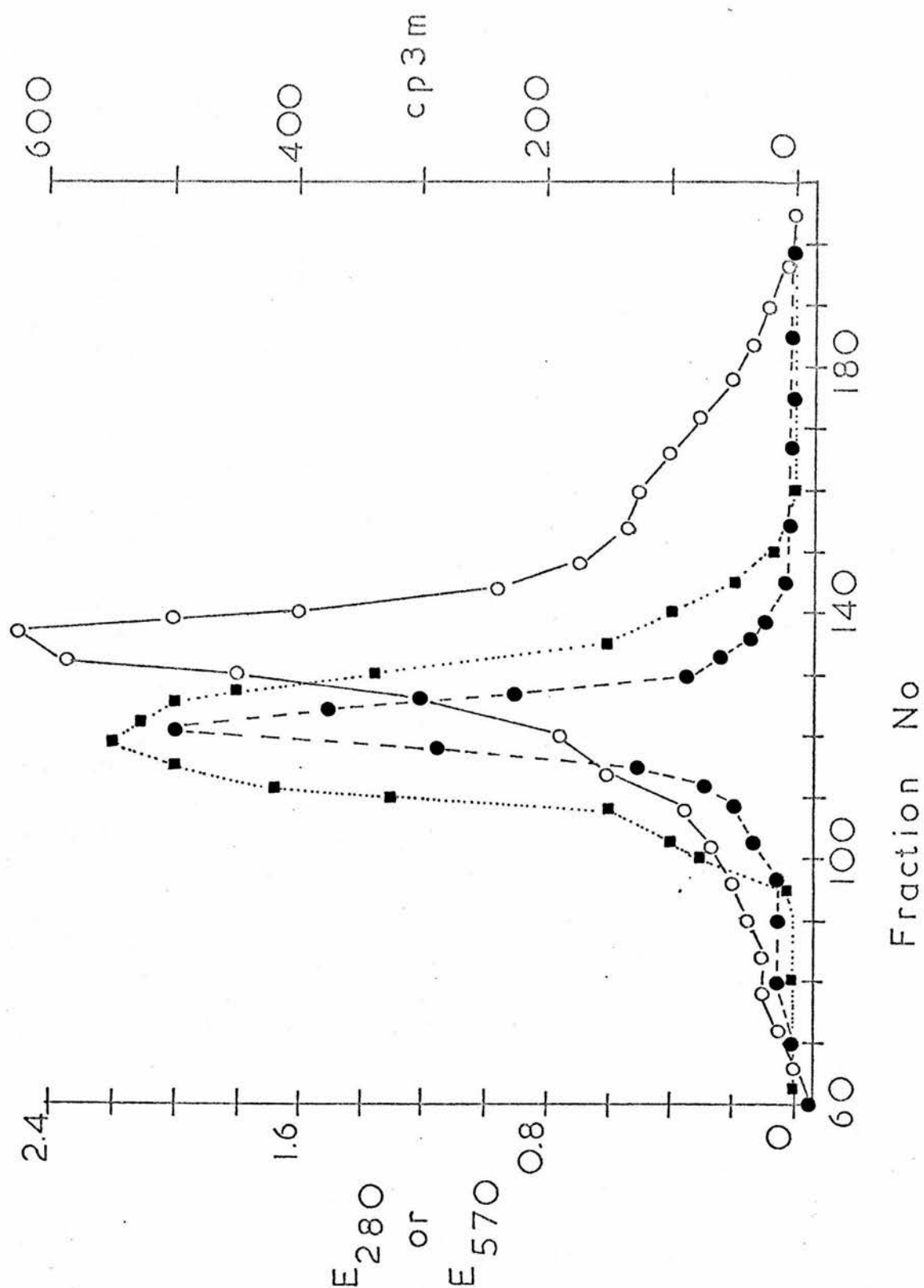


Fig. 30. Chromatography of the large scale subtilisin digest of inhibited pepsin C on Sephadex G-25 (3 x 45 cm), equilibrated with 5% acetic acid.

- - E₂₈₀
-■..... - radioactivity (cp3m)
- - ninhydrin assay (E₅₇₀)

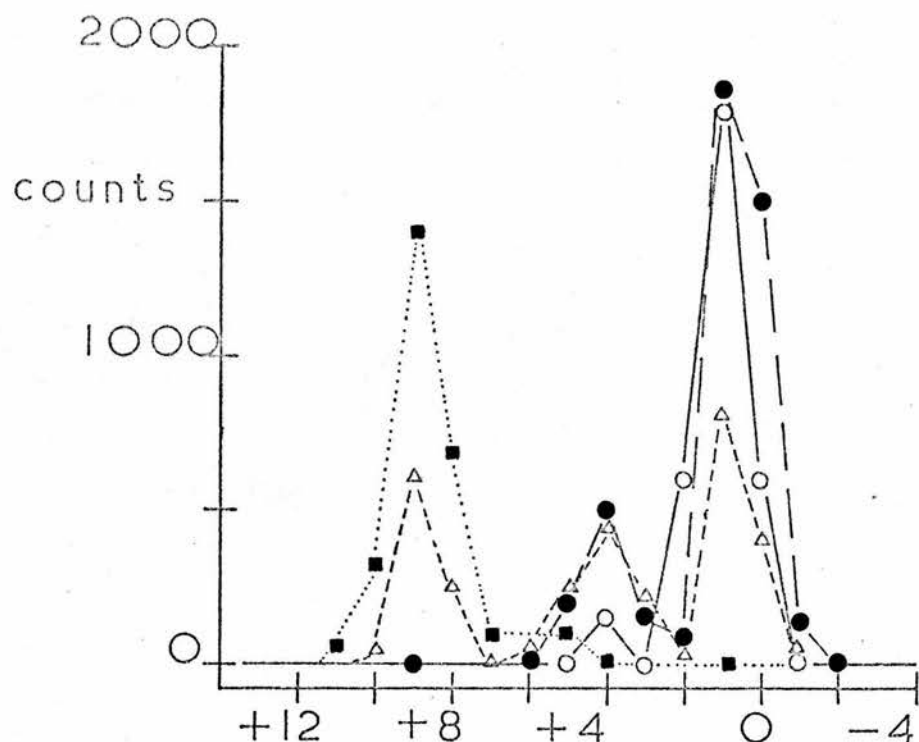


Fig. 31. Electrophoresis at pH 3.6 of fractions obtained from the subtilisin digest.

—○— - Fraction 103 (cp16hr)
 ---●--- - Fraction 110 (cp16hr)
 ---△--- - Fraction 116 (cp16hr)

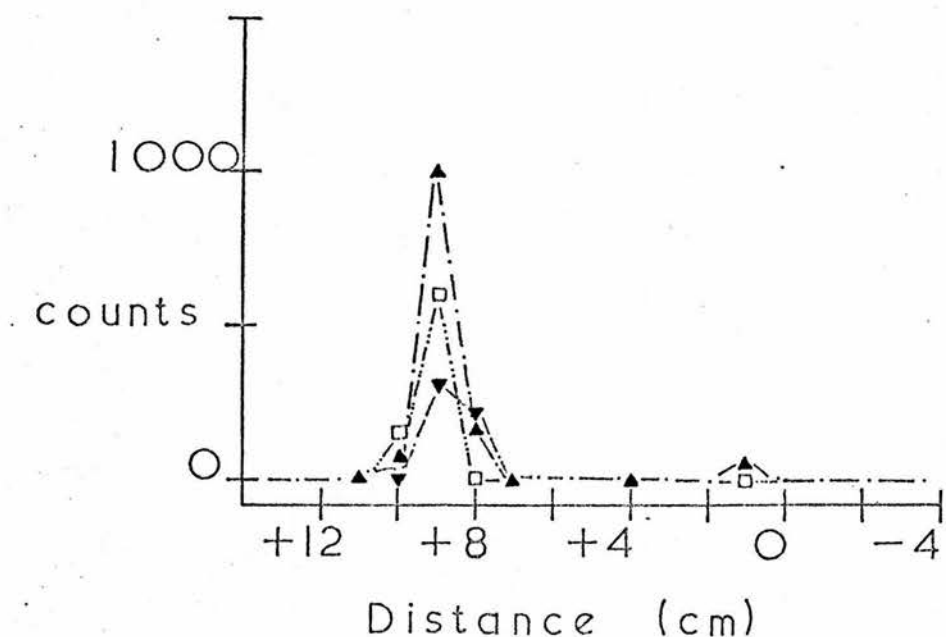


Fig. 31.■..... - Inhibitor breakdown products (cp5m)
 ---▲--- - Fraction 121 (cp8hr)
□.... - Fraction 128 (cp8hr)
 ---▼--- - Fraction 134 (cp16hr)

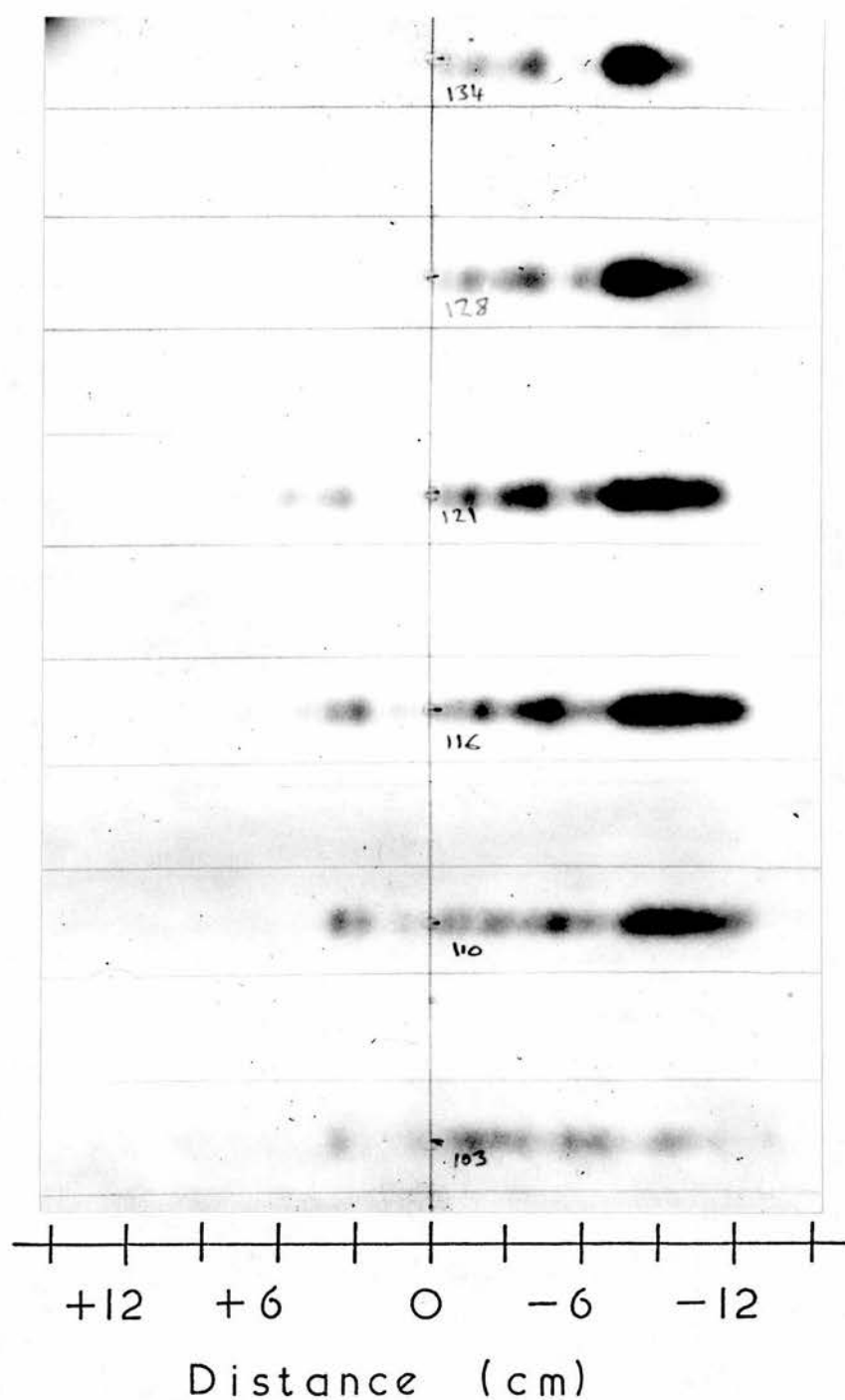


Fig. 32. Ninhydrin staining material obtained after electrophoresis at pH 3.6 of Fractions 103, 110, 116, 121, 128 and 134 from the subtilisin digest.

10. Large scale degradation of inhibited pepsin C
by subtilisin.

Inhibited pepsin C (410 mg) was dissolved in 10 ml of 0.1M ammonium bicarbonate. Subtilisin (4 mg) was added to this and the mixture was incubated for 6 hours at 37° with gentle shaking. At the end of incubation some insoluble material was noticed to be present. This was centrifuged off and washed with 5% acetic acid. The supernatant was acidified to pH 2.3 and the washings were added to this. No further precipitation occurred on standing overnight. The insoluble material was dissolved in 90% formic acid and counted for radioactivity. This showed that about 5% of the total radioactivity was in the insoluble material.

The supernatant and washings were applied to a column of Sephadex G-25 (3 x 45 cm), equilibrated with 5% acetic acid. Fractions (2 ml) were collected, 0.02 ml of each was counted for radioactivity and 0.01 ml was taken for ninhydrin assay. The elution profile is shown in Fig. 30. The first 60 fractions did not contain anything and are not presented in the diagram. A very poor separation was achieved by this chromatography.

Samples were taken from fractions 103, 110, 116, 121, 128 and 134 and subjected to electrophoresis at pH 3.6 for 110 minutes at 1.8 kV to see what pattern of peptides occurred across the radioactive peak. The paper was scanned for radioactivity and then stained with ninhydrin. Fig. 31 shows the distribution of radioactive material with inhibitor breakdown products for comparison and Fig. 32 shows the

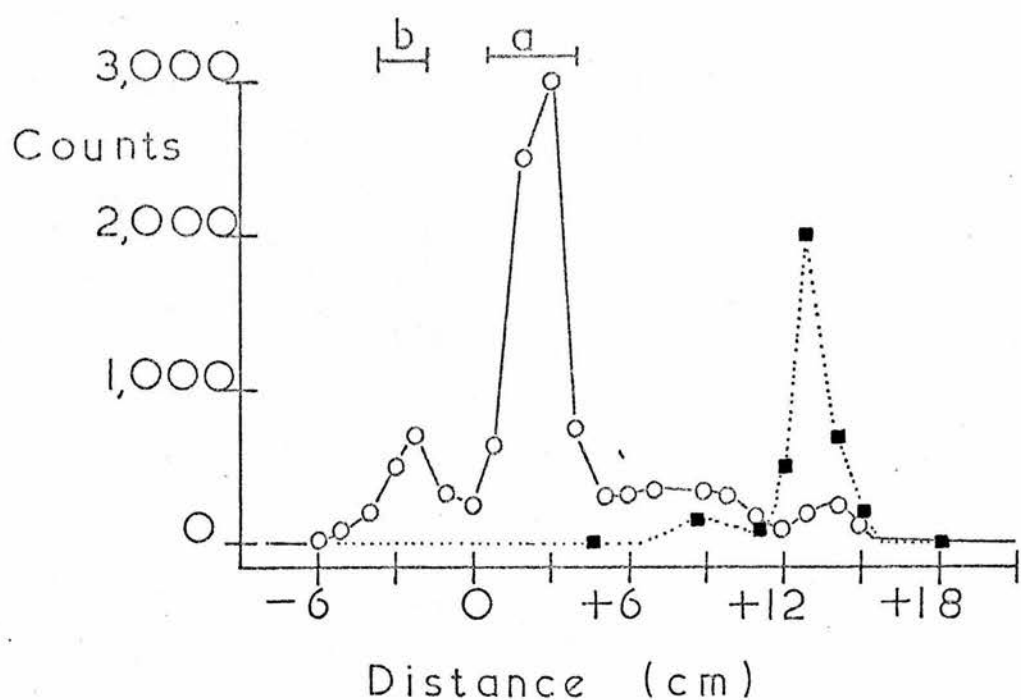


Fig. 33. Electrophoresis at pH 3.6 of fraction S 1

—○— - Fraction S 1 (cp30m)
■..... - Inhibitor breakdown products (cp5m)

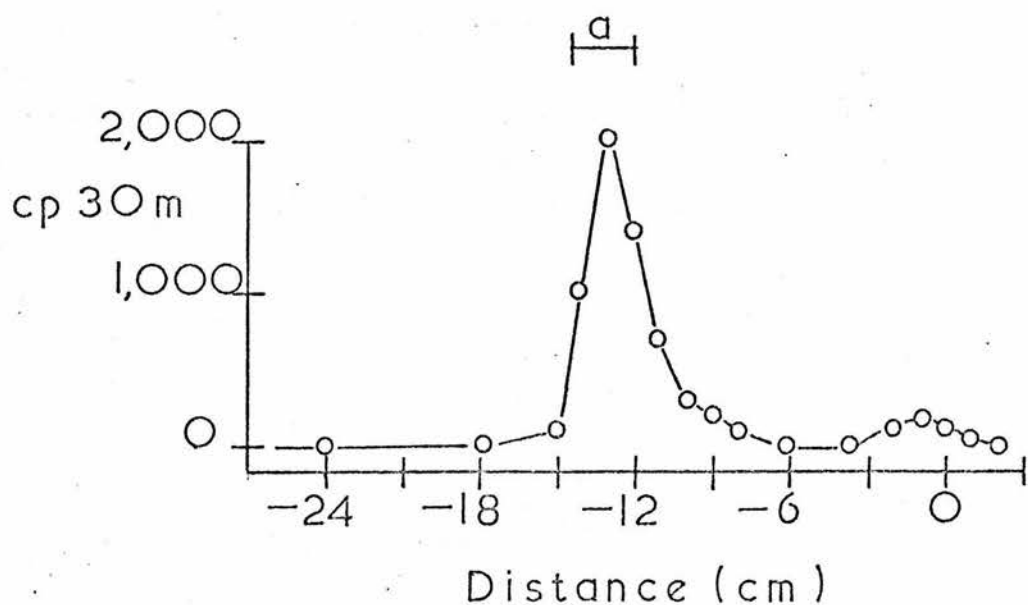


Fig. 34. Electrophoresis at pH 2.0 of S 1a obtained from the pH 3.6 run.

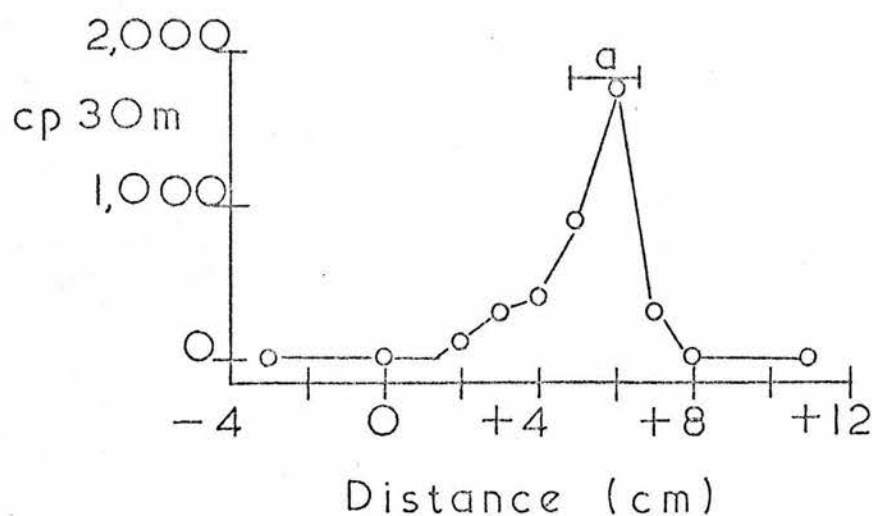


Fig. 35. Electrophoresis at pH 3.6 of S 1a obtained from the pH 2.0 run.

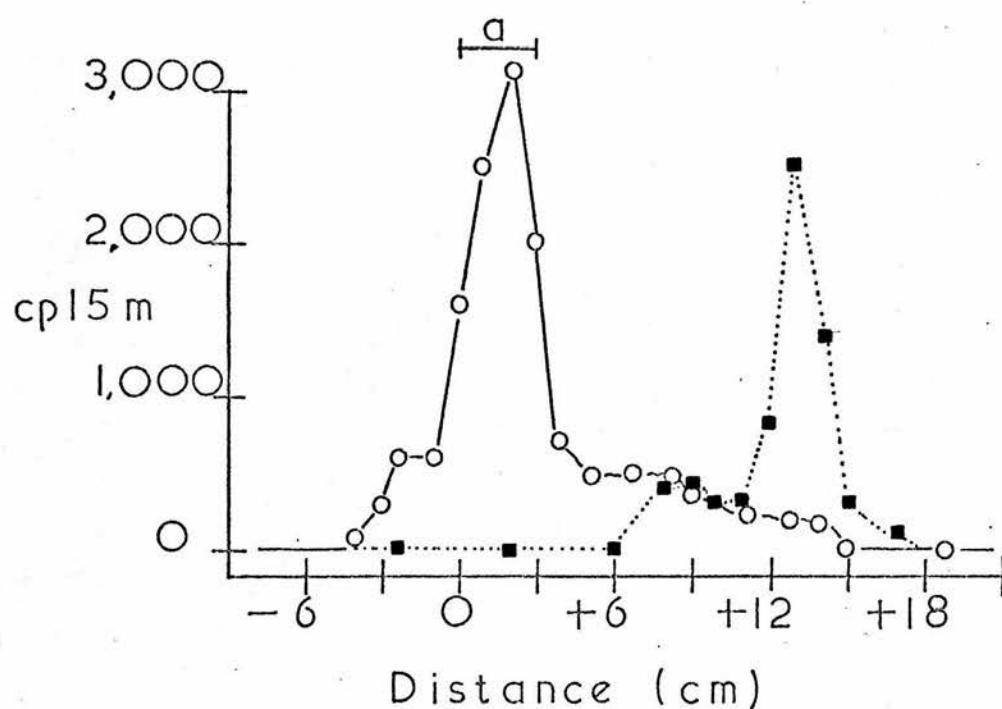


Fig. 36. Electrophoresis at pH 3.6 of fraction S 2.

—○— - Fraction S 2
■..... - Inhibitor breakdown products

ninhydrin staining material. Most of the non-labelled peptides appear to be migrating away from the labelled material.

Fractions were pooled as follows:-

101-105	designated	S 1
106-110	"	S 2
111-124	"	S 3
125-128	"	S 4
129-142	"	S 5

10.1 Separation of S 1.

This fraction was partially dried down and applied in a 4 inch strip to Whatman No. 1 paper with inhibitor breakdown products as a marker. The paper was subjected to electrophoresis at pH 3.6 at 1.8 kV for 2 hours. After drying, the paper was scanned (Fig. 33). Band a was cut out, stitched into a fresh sheet of paper and re-run at pH 2.0 at 2 kV for 1.5 hours. The scan is shown in Fig. 34. Staining a guide strip with ninhydrin showed that the radioactive peak overlapped the leading edge of a long streak of peptides. Therefore, band a was cut out, stitched into a fresh sheet of paper and re-run at pH 3.6 at 2 kV for 6 hours. Fig. 35 shows the scan obtained. Band a was eluted with 5% acetic acid and after hydrolysing a sample for 24 hours amino acid analysis showed it to have a composition of:-

Asp_{1.1} Val_{0.7} Nle_{1.0}.

Determination of the N-terminal residue by DNS-Cl showed DNS-valine.

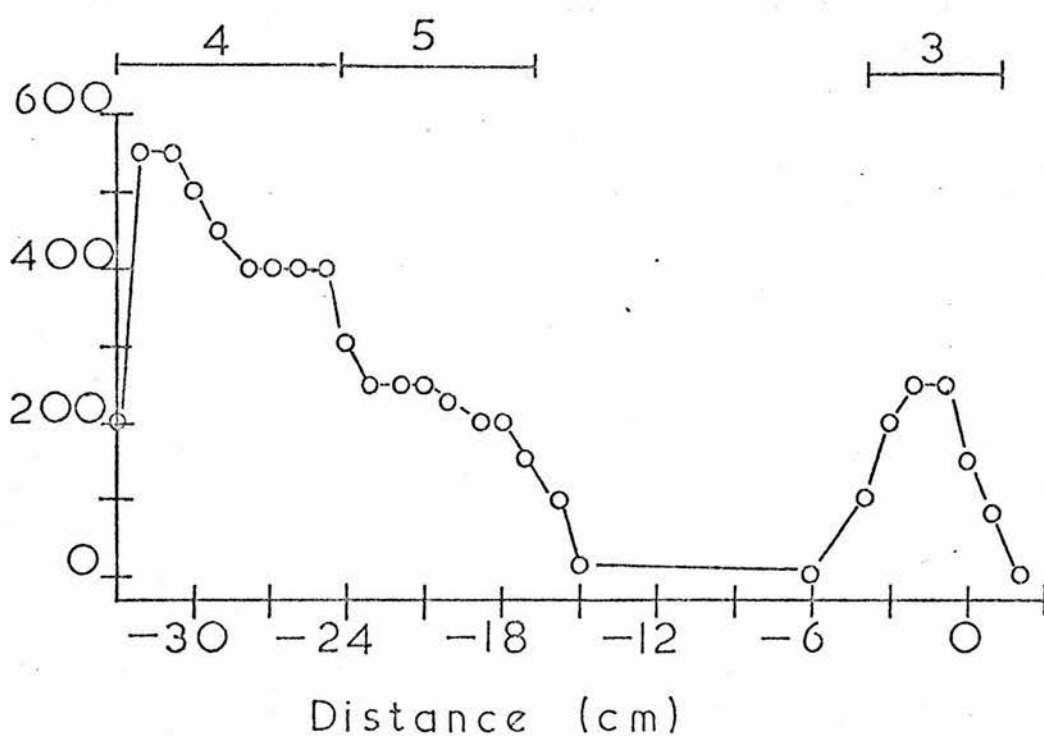
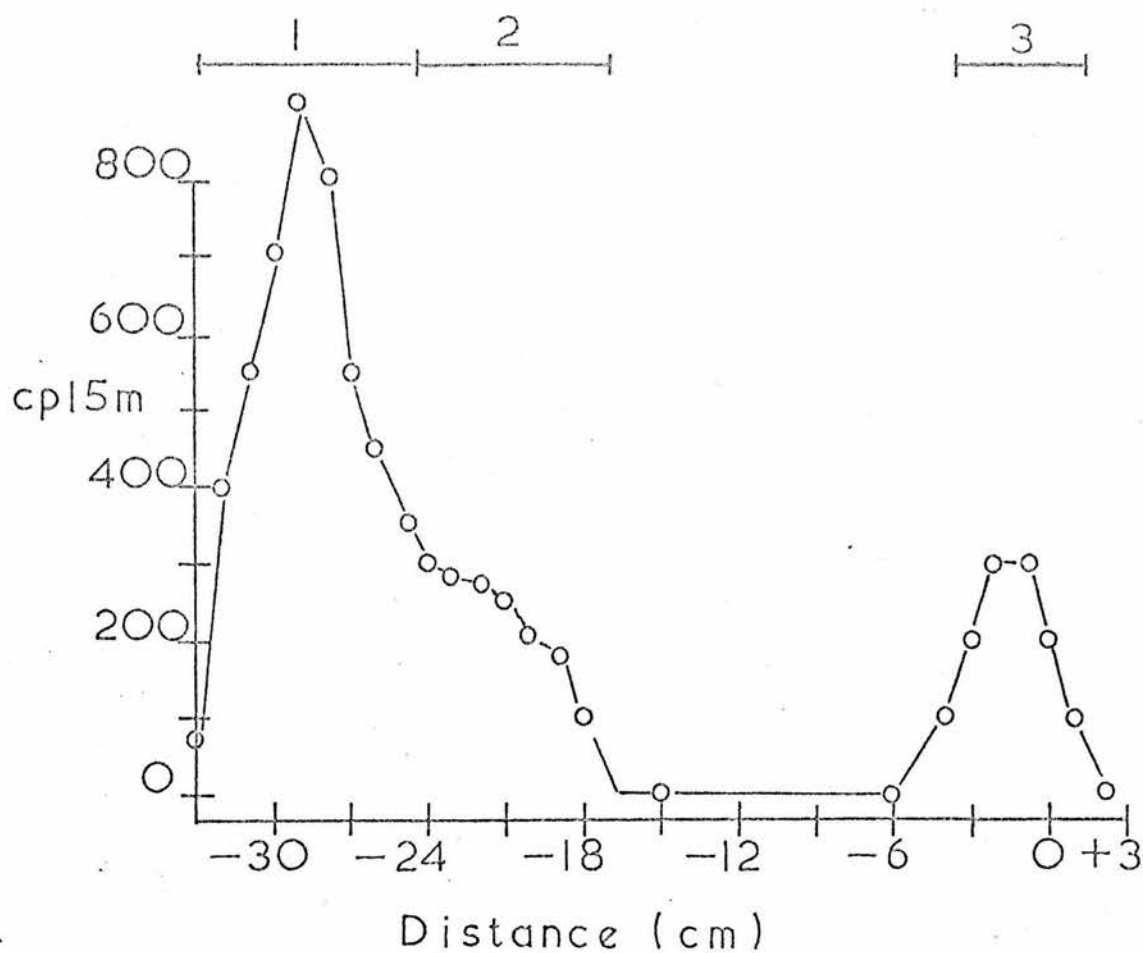


Fig. 37. Electrophoresis at pH 2.0 of S 2a obtained from the pH 3.6 run. (The diagram is divided into two to show that the material did not migrate uniformly across the paper).

This must be Val-Asp, the norleucine being derived from the inhibitor.

Band S 1b was re-run at pH 2.0 and the radioactive band from this was re-run at pH 6.5. After scanning, the radioactive band obtained was eluted with 5% acetic acid and hydrolysed. Amino acid analysis showed the presence of a large excess of the other amino acids in relation to norleucine, as might be expected from a band from this neutral region.

10.2 Separation of S 2.

This fraction was partially dried down and applied in a 4 inch strip to No. 1 paper with inhibitor breakdown products as a marker. The paper was subjected to electrophoresis at pH 3.6 at 1.8 kV for 2 hours. After drying, the paper was scanned (Fig. 36). Band 2a was cut out, stitched into a fresh sheet of paper and re-run at pH 2.0 at 2 kV for 4 hours.

Scanning this strip revealed that the material had not migrated uniformly across the 4 inch strip. Fig. 37 shows the scans for both halves of the strip separately. From this, bands 1-5 designated as shown were eluted from the paper with 5% acetic acid.

Band 3 was hydrolysed for 24 hours and amino acid analysis showed a very large proportion of most of the other amino acids in relation to norleucine.

Bands 1, 2, 4 and 5 were applied separately to No. 1 paper in half inch strips and subjected to chromatography in n-butanol:n-butyl acetate:acetic acid:water: :19:1:5:25

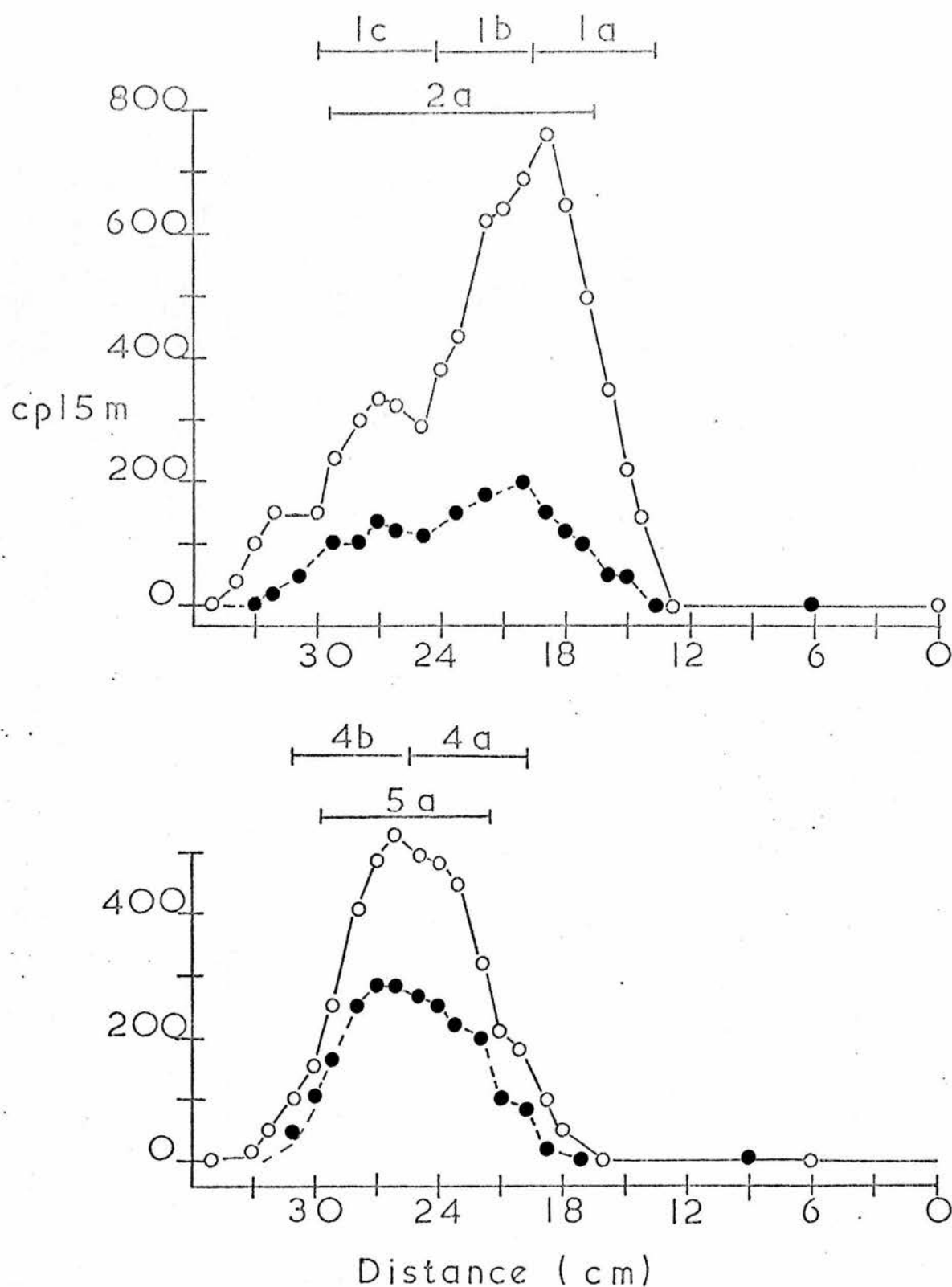


Fig. 38. Chromatography in n-butanol:n-butyl acetate:
acetic acid:water::19:1:5:25 of fractions

—○— - S 2a1)
 ---●--- - S 2a2) — top
 —○— - S 2a4)
 ---●--- - S 2a5) — bottom

obtained from the pH 2.0 electrophoresis.

for 13 hours. The papers were dried and scanned (Fig. 38).

Fractions 1a, 1b, 1c, 2a, 4a, 4b and 5a, as shown by the bars, were eluted from the papers with 5% acetic acid. Samples were taken from each fraction for N-terminal analysis by DNS-Cl and the remainder of the fractions were hydrolysed for 24 hours. The analyses are given below, the ratios of the amino acids being calculated relative to norleucine = 1.0:-

	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Nle	Phe
1a	2.7	0.5	0.9	1.3	0.8	1.6	0.6	1.0	2.2	2.8	1.0	0.7
4a	1.6	0.3	0.3	0.7	-	0.6	0.2	0.8	1.2	1.0	1.0	0.4
1b	1.1	-	-	-	-	-	-	0.7	0.8	0.6	1.0	-
1c	1.1	-	-	-	-	-	-	0.7	0.7	0.7	1.0	-
4b	1.1	-	-	-	-	-	-	0.7	0.6	0.5	1.0	-
2a	2.6	0.5	1.2	1.9	1.9	1.9	0.8	1.2	1.9	2.4	1.0	1.2
5a	1.7	0.2	0.4	0.7	0.3	0.7	0.3	0.9	1.1	0.9	1.0	0.5

N-terminal analysis on 1a, 2a, 4a and 5a showed DNS-leucine as the major spot but also smaller amounts of DNS-glutamate, DNS-glycine and DNS-alanine. These fractions cannot have been completely purified.

Dansylation of 1b, 1c and 4b, on the other hand, showed only DNS-leucine. Since these peptides had only been hydrolysed for 24 hours this might explain the low recoveries of valine, isoleucine and leucine but it appears that 1b, 1c and 4b are all derived from the same peptide:-

Leu-(Ile,Val,Asp)

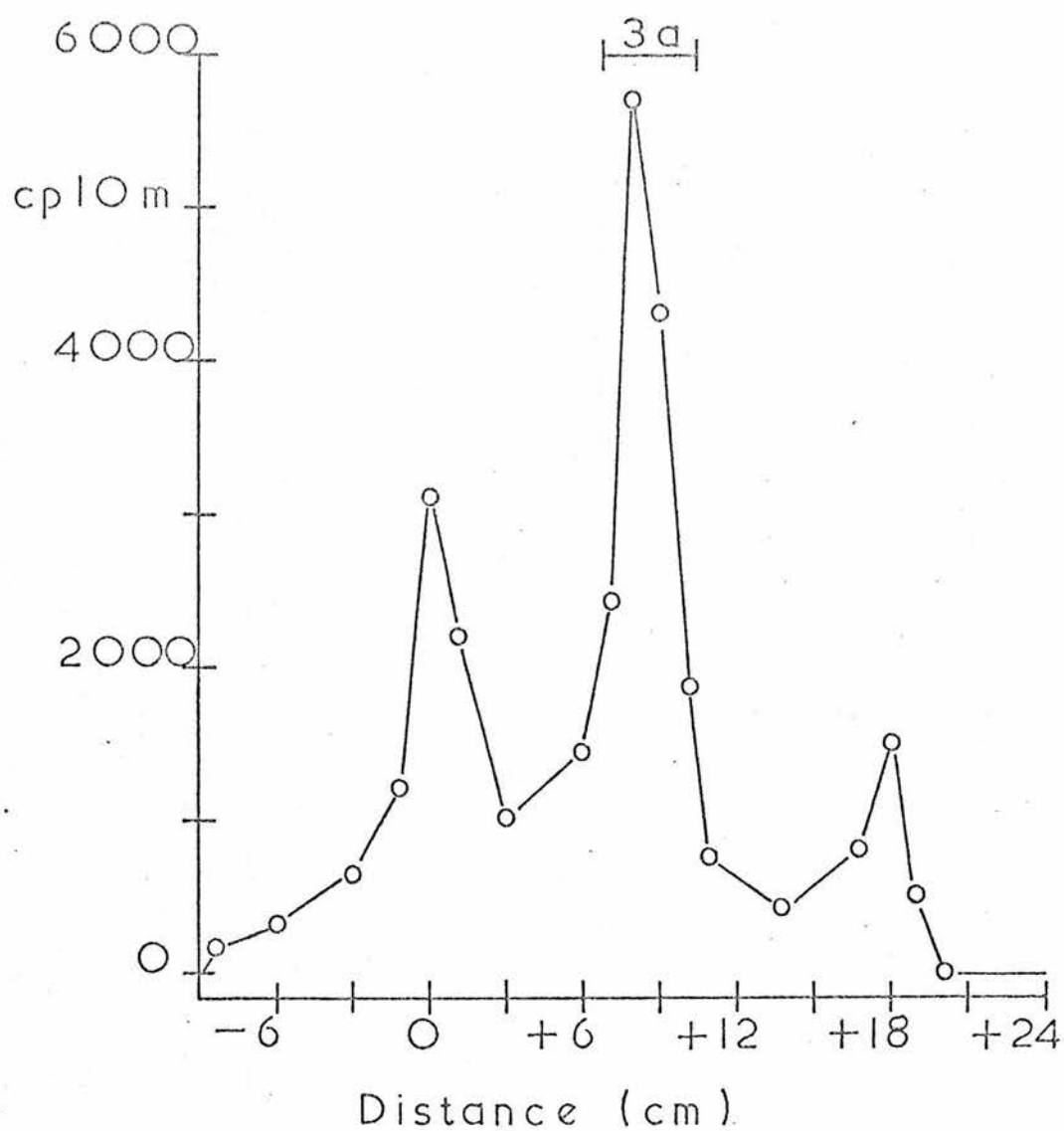


Fig. 39. Electrophoresis at pH 3.6 of fraction S 3

This peptide may also be contained in the other fractions since 1a, 2a, 4a and 5a all have at least 1 residue of aspartate, valine, isoleucine and leucine but other non-labelled peptide(s) are also present, shown by the heterogeneity of the N-terminal analysis.

If peptide S 2alb is derived from the same labelled site as peptide S 1a, then the sequence:-

Leu-Ile-Val-Asp or Leu-Val-Asp-Ile

can be established by overlapping. Presumably the label is attached to the aspartate residue as it is difficult to see how DNM could attach itself to the hydrophobic side-chains of the valine, isoleucine or leucine, although it might conceivably be attached to the α carboxyl group of the peptide.

10.3 Separation of S 3.

This fraction was taken to dryness in vacuo and a large amount of solid material, yellow in colour, was left at the bottom of the vial. This material re-dissolved in 0.5 ml of 5% acetic acid and on application of this to No. 1 paper in a 4 inch strip a brilliantly yellow coloured strip, looking as if it contained a lot of salt, was obtained. When the paper was subjected to electrophoresis at pH 3.6 it burned through at one side of the yellow strip and scanning revealed that the radioactive material had not migrated. This strip around the origin was cut out, stitched into a fresh sheet of paper and run again at pH 3.6 at 1.9 kV, when the pattern shown in Fig. 39 was obtained. The band at the origin was ignored since some of the original yellow colour was still there, although most of it had migrated. Presumably,

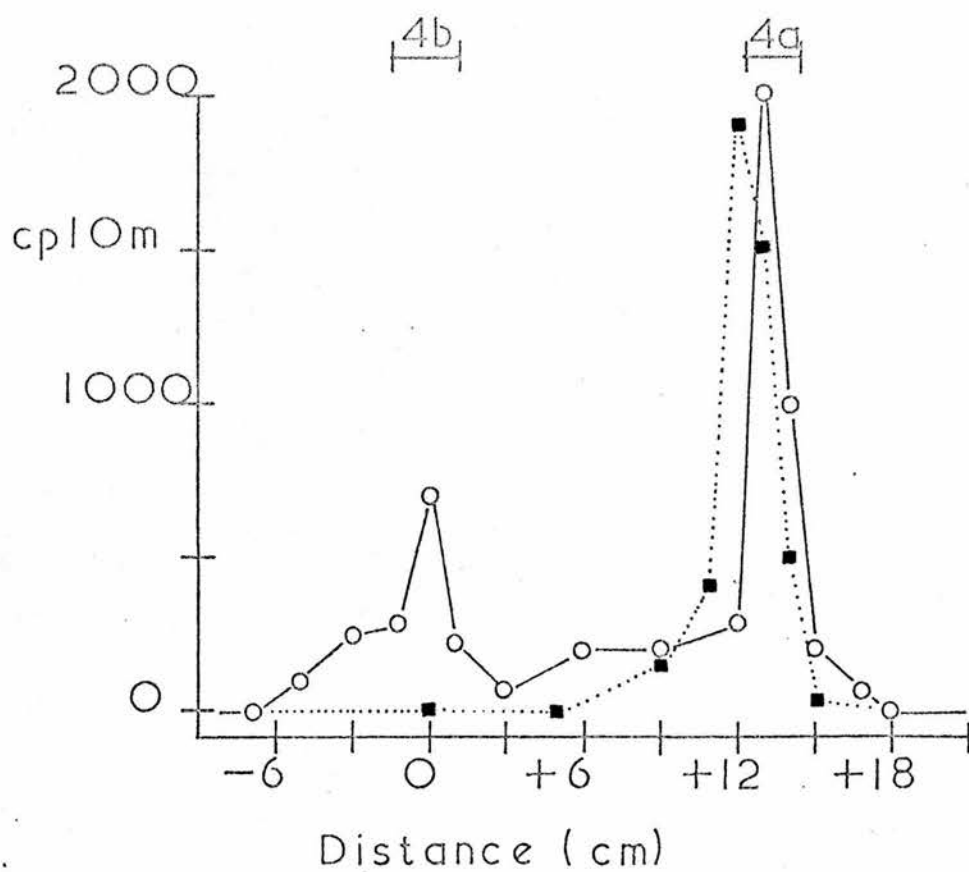
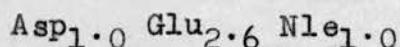


Fig. 40. Electrophoresis at pH 3.6 of fraction S 4.

—○— - Fraction S 4
■..... - Inhibitor breakdown products

when the paper was burned through, some material became irreversibly adsorbed on to the paper.

Band 3a was eluted from the paper with 5% acetic acid. Amino acid analysis on a fraction of this material, after 24 hours hydrolysis, gave the composition:-



The remainder of the material was re-applied to No. 1 paper in a 2 inch strip and subjected to electrophoresis at pH 6.5. The radioactive band from this was cut out and subjected to paper chromatography in:-

t-butanol:methyl ethyl ketone:water::2:2:1.

The radioactive band from this (R_F 0.75) was eluted with 5% acetic acid. A sample was hydrolysed for 24 hours and amino acid analysis showed it to contain only norleucine. N-terminal analysis by DNS-Cl gave no spots and so this fraction must be some derivative of norleucine formed by the fission of one of the inhibitor bonds to the enzyme.

This fraction may contain the ammonium bicarbonate in which the subtilisin digest was carried out (accounting for the large amount of salt) since ninhydrin assay (Fig. 30) shows the peak of ninhydrin colour is in this fraction.

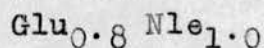
10.4 Separation of S 4.

This material was dried down in vacuo, re-dissolved in 5% acetic acid and applied to No. 1 paper in a 4 inch strip with inhibitor breakdown products as marker. The paper was subjected to electrophoresis at pH 3.6 at 1.9 kV for 2 hours, dried and scanned (Fig. 40).

Not too much attention was paid to fraction 4b because of the low yield of radioactive material but, after re-running the band at pH 2.0, elution and amino acid analysis showed the presence of a large excess of the other amino acids in relation to norleucine.

Fraction 4a, on the other hand, migrated very similarly to the main inhibitor breakdown products peak. Hydroxyacetyl norleucine ester should not migrate at this pH but the unesterified material might. Since subtilisin has an esterase activity (Hagihara 1960) and leucine methyl ester is a fairly good substrate to undergo this reaction, 2 samples of norleucine methyl ester were incubated in ammonium bicarbonate at 37° for 4 hours, one with and one without subtilisin. Electrophoresis of the two mixtures at pH 3.6 with norleucine and norleucine ester as markers showed that BOTH, after 4 hours incubation, had more norleucine than norleucine ester. Apparently the pH of ammonium bicarbonate must be high enough to remove the ester group from the norleucine ester without the esterase activity of subtilisin enhancing this effect.

Amino acid analysis on a portion of fraction 4a showed it to have the composition:-



This fraction could either have the norleucine attached to the glutamate or could simply be hydroxyacetyl norleucine (presumably the major product formed by fission of an inhibitor bond to the enzyme) contaminated with either free glutamate or glutamyl-glutamate. A check on the mobilities

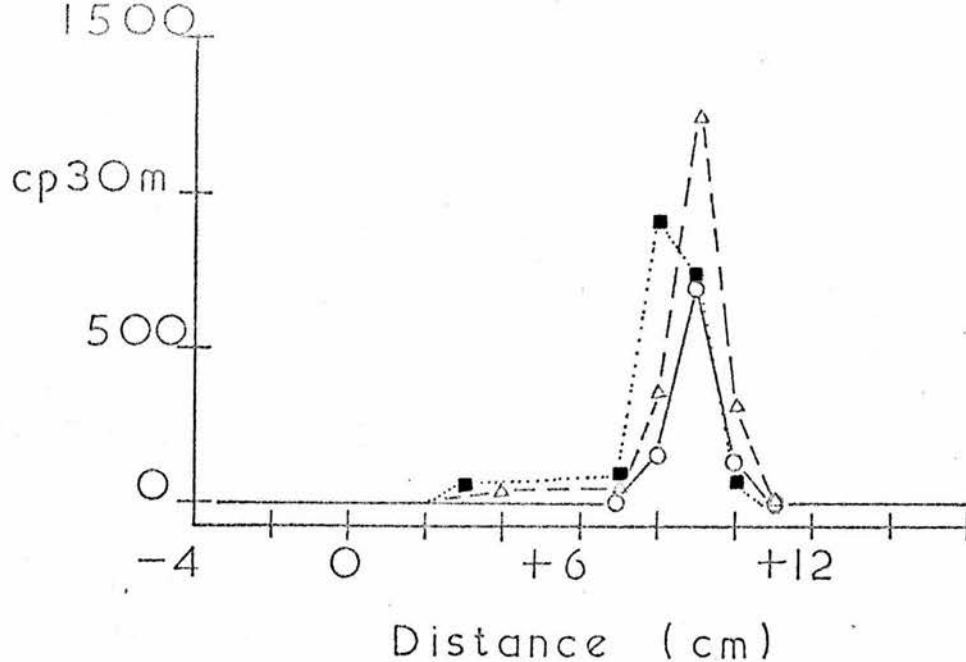


Fig. 41. Electrophoresis at pH 3.6 of fraction S 4a.

- - Fraction S 4a
- △--- - Fraction S 4a + Inhibitor breakdown products
-■..... - Inhibitor breakdown products

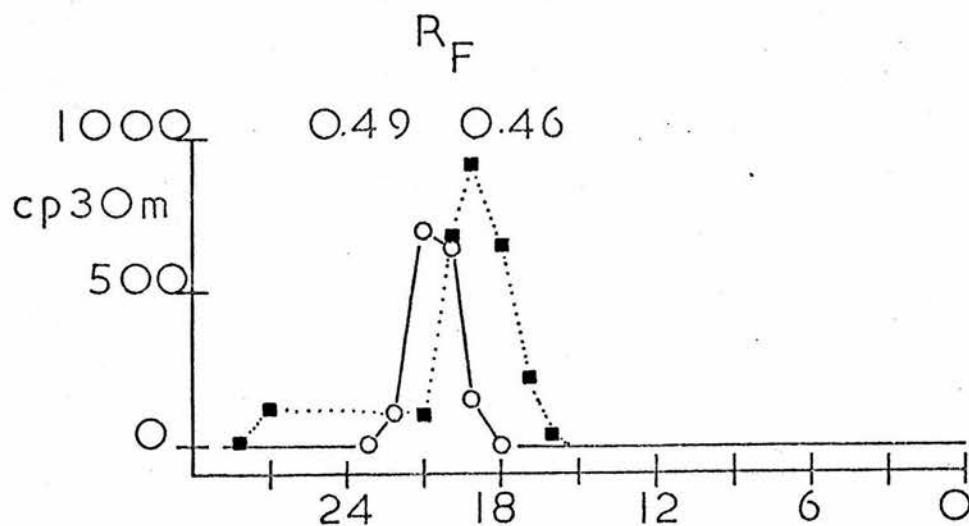


Fig. 42. Chromatography in pyridine:isoamyl alcohol: water::7:7:6 of fraction S 4a

- - Fraction S 4a
-■..... - Inhibitor breakdown products

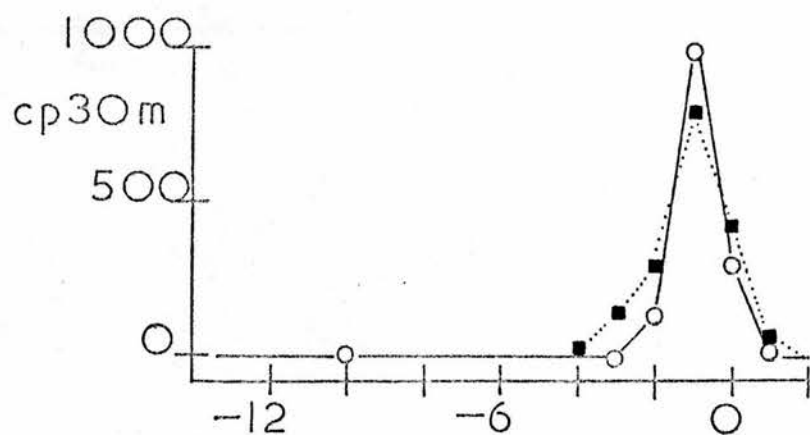


Fig. 42. Electrophoresis at pH 2.0 of fraction S 4a.

—○— - Fraction S 4a
■..... - Inhibitor breakdown products

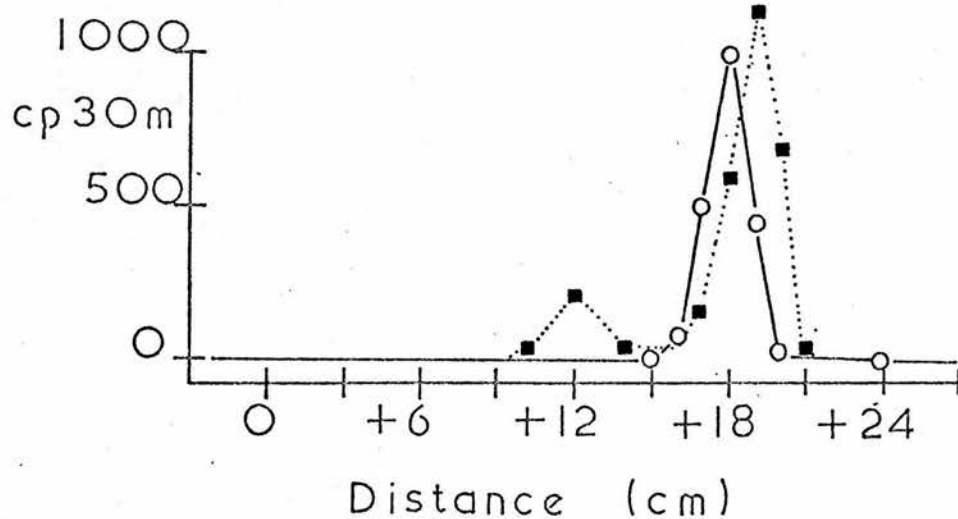


Fig. 42. Electrophoresis at pH 6.5 of fraction S 4a.

—○— - Fraction S 4a
■..... - Inhibitor breakdown products

of glutamate and glutamyl-glutamate at this pH showed that they did not migrate anywhere near the position of this band.

Fig. 41 shows that a mixture of 4a and inhibitor breakdown products did not separate on electrophoresis at pH 3.6. Fig. 42 shows that 4a migrated very similarly to the inhibitor breakdown products at pH 2.0, 6.5 and on chromatography in pyridine:isoamyl alcohol:water: :7:7:6.

Elution and amino acid analyses of the labelled bands obtained from these treatments showed that after electrophoresis at pH 2.0 the glutamate was still present whereas, after electrophoresis at pH 6.5 and after chromatography in pyridine:isoamyl alcohol:water, there was no glutamate present. This must mean that either the glutamate containing derivative migrates differently from the norleucine containing derivative on electrophoresis at pH 6.5 and on chromatography but not at pH 2.0 or, assuming the glutamate is bound to the norleucine, treatment at pH 6.5 or chromatography in this system splits this bond, whereas treatment at pH 2.0 does not.

Offord (1966) has shown how to relate the relative mobilities of peptides to net charge and molecular weight. This was used to work out whether the norleucine derivative could be attached to the glutamate in this fraction.

If the mobilities, m , (corrected for endosmosis by including a neutral marker) of inhibitor breakdown products (presumably hydroxyacetyl norleucine $M = 189$) and 4a (assuming it to be glutamyl- γ -hydroxyacetyl norleucine $M = 318$) at pH 6.5 and 3.6 are compared:-

	pH 6.5		pH 3.6		
	m	corrected	m	corrected	
hydroxyacetyl norleucine	+19	+23	+8	+10	cm
Glu- γ -hydroxyacetyl Nle	+18	+22	+9	+11	cm

Allowing that, at pH 6.5, hydroxyacetyl norleucine has charge -1, then at pH 3.6 it must have a charge of $\frac{10}{23} = -0.44$ (and therefore $pK_a = 3.71$)

Also, the charge on glutamyl- γ -hydroxyacetyl norleucine at pH 6.5 (according to Offord) = $-1 \times \frac{22}{23} \times \left(\frac{318}{189}\right)^{2/3}$
 $= -1.23$

and the charge at pH 3.6 = $-1 \times \frac{11}{23} \times \left(\frac{318}{189}\right)^{2/3}$
 $= -0.68.$

Therefore, at pH 6.5, if one allows

-1 for the norleucine carboxyl and -1 for the glutamate carboxyl,
 there is $-1.23 - (-2) = +0.77$ extra charge which could be contributed by the amino group which would have to have a pK_a of $6.5 - \log \frac{0.23}{0.77} = 7.02.$

At pH 3.6, if one allows

-0.44 for the norleucine carboxyl ($pK_a = 3.71$)
 and +1.0 for the amino group ($pK_a = 7.02$),
 then the remaining carboxyl must have
 $-0.68 - (+1 - 0.44)$
 $= -1.24$ charges.

Thus, although it is unlikely but not impossible to have an amino group with a pK_a of 7.02, it is not possible to have a carboxyl group with 1.24 charges. Fraction 4a cannot be

glutamyl- γ -hydroxyacetyl norleucine but would seem to be hydroxyacetyl norleucine with contaminating glutamate (from another peptide ?).

As a last effort to find how this glutamate was involved, a portion of 4a was applied to a column of Zeo-Karb 225 (equilibrated with 0.1N-formic acid) at pH 2 and washed through at pH 2, collecting 1 ml fractions. The eluant was changed to pyridine:acetic acid, pH 5, and after about 40 ml of this had been run through the eluant was changed to 0.1N-ammonia. Assaying the fractions for radioactivity revealed that the isotope only occurred in the first three fractions (i.e. it had run straight through the column). Hydrolysis and amino acid analysis of these pooled fractions showed that the glutamate content had dropped to 0.4 residues in relation to norleucine = 1.0. Since the material was applied to a cation exchanger at pH 2, if 4a was glutamyl- γ -hydroxyacetyl norleucine, it should have been bound to the resin, whereas one would not expect hydroxyacetyl norleucine to be bound. This separation was not as satisfactory as it could have been, but it seems that fraction S 4a contains hydroxyacetyl norleucine i.e. an inhibitor residue which has become detached from the protein.

10.5 Separation of S 5.

This material was dried down in vacuo, re-dissolved in 0.5 ml of 5% acetic acid and applied in a 4 inch strip to No. 1 paper with inhibitor breakdown products as a marker. The paper was subjected to electrophoresis at pH 3.6 at 2 kV for 2 hours. After drying, the paper was scanned (Fig. 43).

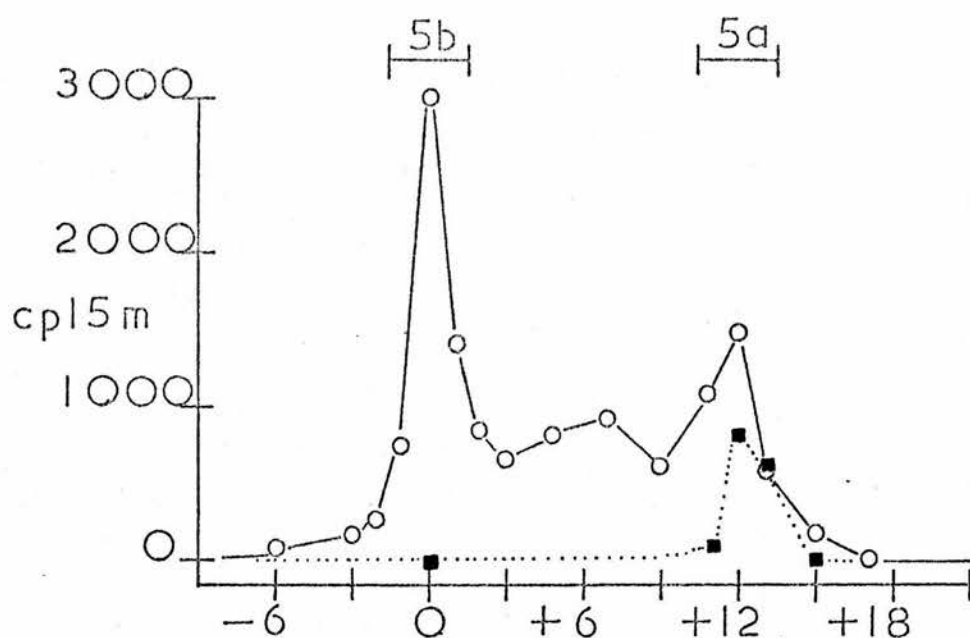


Fig. 43. Electrophoresis at pH 3.6 of fraction S 5

—○— - Fraction S 5
■..... - Inhibitor breakdown products

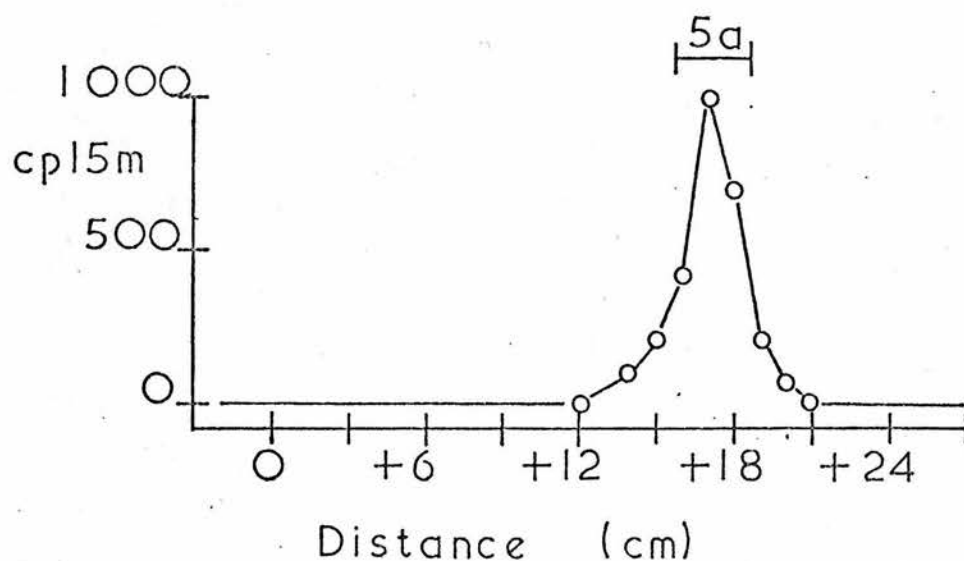
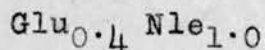


Fig. 44. Electrophoresis at pH 6.5 of S 5a obtained from the pH 3.6 run.

Band 5a was cut out, stitched into a fresh sheet of paper and re-run at pH 6.5 (Fig. 44). The radioactive band from this was eluted and, after hydrolysis, amino acid analysis showed the composition to be



It seems likely that this fraction, similarly to 4a, also contains hydroxyacetyl norleucine produced by fission of an inhibitor bond to the enzyme.

Fraction 5b was cut out and re-run at pH 3.6. Again the material had not moved from the origin so this band was stitched into a fresh sheet, run at pH 2.0 and the material had barely moved at this pH either. This suggests either a very large peptide (unlikely from its elution from G-25) or that the peptide(s) had become adsorbed on to the paper. This latter idea was made more creditable as it proved very difficult to elute the material (several washings were needed) from the paper. Amino acid analysis of the eluted material showed the composition (relative to norleucine = 1.0):-

	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Nle	Tyr	Phe
5b	1.0	2.9	3.3	4.0	1.5	3.4	1.0	1.7	1.6	2.2	1.0	6.0	4.2

N-terminal analysis by DNS-Cl showed DNS-leucine, DNS-proline, DNS-alanine, DNS-glycine and DNS-threonine/glutamate. This fraction has not been very well purified.

10.6 Conclusions from the subtilisin digest.

Since most of the radioactive label from the inhibited enzyme was found in fractions which contained only one amino

acid, norleucine, under the conditions used for this digestion and series of separations, it was decided to degrade the inhibited pepsin C under acidic conditions in the hope that the enzyme-inhibitor bonds would be stable under these conditions.

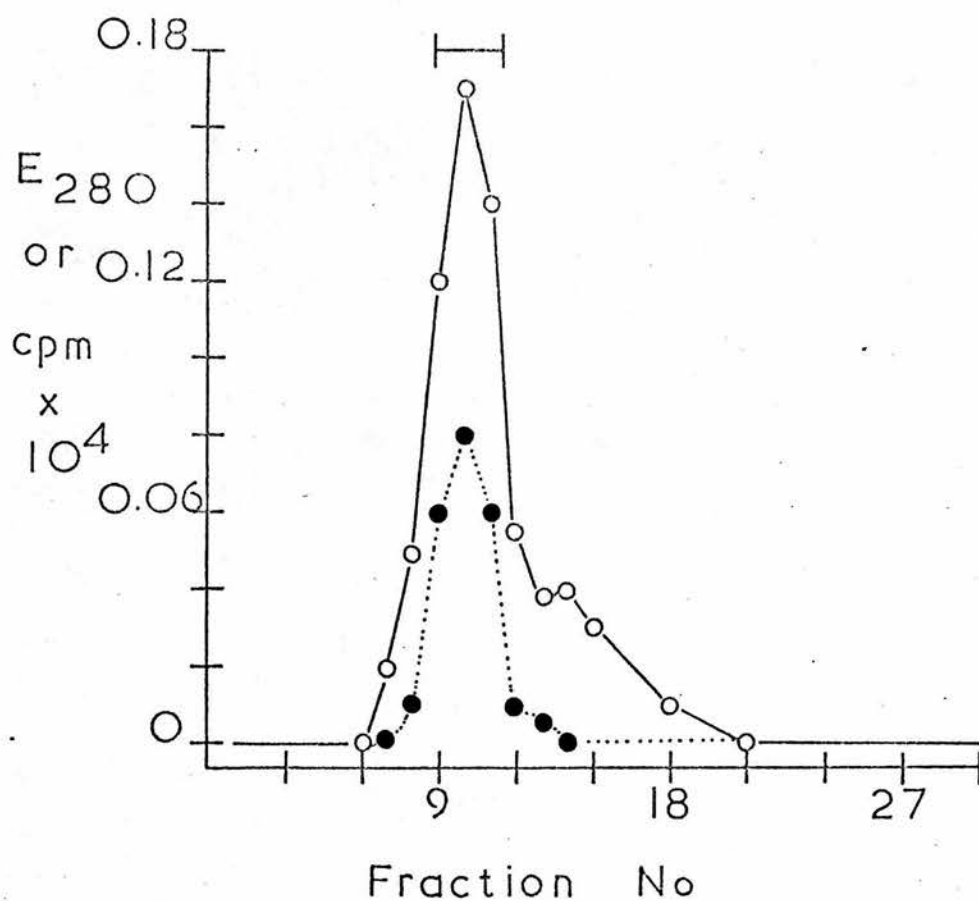


Fig. 45. Chromatography of the soluble (in 5% acetic acid) material obtained from cyanogen bromide fission of inhibited pepsin C on Sephadex G-150 (1 x 40 cm), equilibrated with 5% acetic acid.

—○— - E₂₈₀
●..... - radioactivity.

11. Small scale degradation of inhibited pepsin C by cyanogen bromide.

^{14}C -labelled enzyme (15 mg) was dissolved in 0.5 ml of 80% formic acid and to this was added cyanogen bromide (15 mg) in 0.5 ml of 80% formic acid. (Ostoslavskaya et al 1968). The mixture was left at room temperature for 24 hours and then freeze-dried.

5% acetic acid (2 ml) was added to the lyophilised material and the insoluble material left was centrifuged off.

a) Supernatant.

This was applied to a column of Sephadex G-150 (1 x 40 cm), equilibrated with 5% acetic acid and 5 ml fractions were collected. Samples of these were taken for scintillation counting and the elution profile is shown in Fig. 45. A 100% recovery of radioactive material was obtained from the column and fractions 9-11 were pooled.

N-terminal analysis of this material by DNS-Cl showed DNS-glycine as the major spot with a very faint spot of DNS-serine.

A sample was hydrolysed for 24 hours at 105° and, after removal of the hydrochloric acid on the rotary evaporator, the hydrolysate was dissolved in 1 ml of pyridine: acetic acid: water::100:4:1900, pH 6.5, and heated for a further hour at 105° to convert homoserine-lactone into homoserine (R.P. Ambler, personal communication). Amino acid analysis gave the composition (relative to norleucine = 1.0):-

Asp	Thr	Ser	Hsr	Glu	Pro	Gly	Ala	Val	Ile	Leu	Nle	Phe	Lys
1.9	1.7	3.0	1.0	2.8	2.4	3.1	2.8	1.7	0.9	3.1	1.0	0.5	2.0

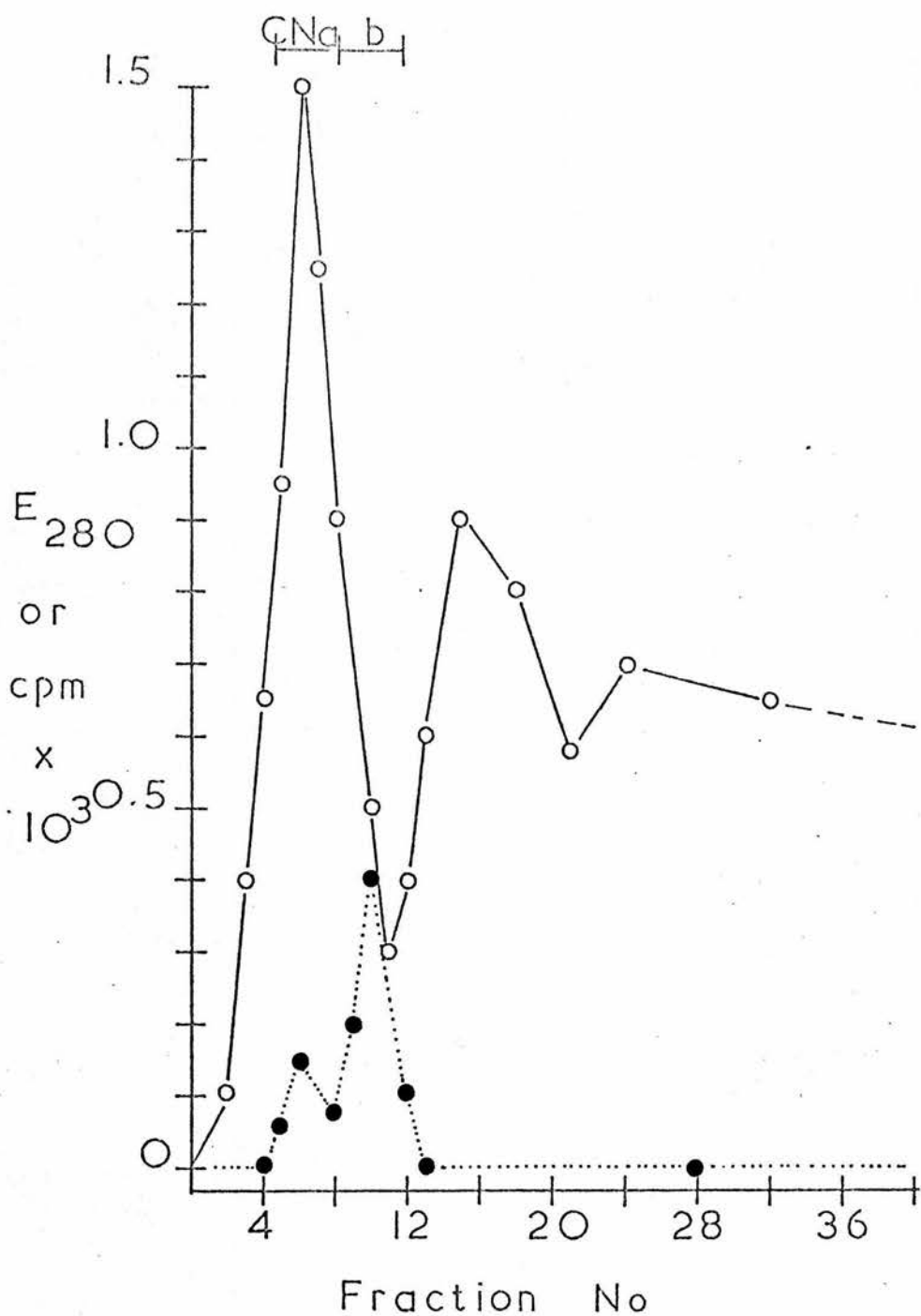


Fig. 46. Chromatography of the soluble (in 80% formic acid) material obtained from cyanogen bromide fission of inhibited pepsin C on Sephadex G-150 (1 x 45 cm), equilibrated with 80% formic acid.

—○— - E_{280}
●..... - radioactivity

This fraction contained 27 amino acids with 1 norleucine:1 homoserine with N-terminal glycine. Treatment with carboxypeptidase A released homoserine and nothing else, so the penultimate residue must be resistant to carboxypeptidase A digestion. After 1 cycle of Edman degradation N-terminal analysis by DNS-Cl gave DNS-proline/valine. Thus this material may be derived from a peptide:-

Gly-Pro/Val-(Asp₂Thr₂Ser₃Glu₃Pro₁₋₂Gly₂Ala₃Val₁₋₂Ile₁Leu₃Phe₁
Lys₂)-Met-

It is interesting to note at this stage that there are no tyrosine residues anywhere near the labelled group in this peptide.

b) Insoluble material.

This was suspended in 5% acetic acid, made 6M in urea but would not dissolve. The pH of this suspension was taken slowly to 6 with dilute ammonia but the material was still insoluble. The solvent was removed on the rotary evaporator, 2 ml of 80% formic acid was added and the material dissolved. This solution was applied to a column of Sephadex G-150 (1 x 45 cm), equilibrated with 80% formic acid, and 5 ml fractions were collected. The elution profile is shown in Fig. 46. Fractions were pooled as indicated.

After hydrolysis, amino acid analysis of fraction CNa showed a very high ratio of all the other amino acids in relation to norleucine so this fraction probably represents the large 'core' of the enzyme.

Fraction CNb had the following amino acid composition:-

Asp	Thr	Ser	Glu	Gly	Ala	Val	Ile	Leu	Nle	Phe
0.4	0.5	0.8	1.2	2.0	0.9	1.2	0.6	1.0	1.0	0.6

and also a large amount of urea.

Several interesting points arise from this,

I) Since there is no homoserine in the fraction, is it derived from the C-terminus of the enzyme?

II) The inhibitor may be bound to some residue other than aspartate, as there are only 0.4 residues of aspartate in relation to 1.0 for norleucine.

III) Again, there is no tyrosine in the analysis.

From this pilot experiment it looked as if cyanogen bromide would be a useful tool to find the site of attachment of one of the labelling groups in the inhibition enzyme by its solubility in 5% acetic acid and the remaining material, because of its insolubility, could be further digested with pepsin.

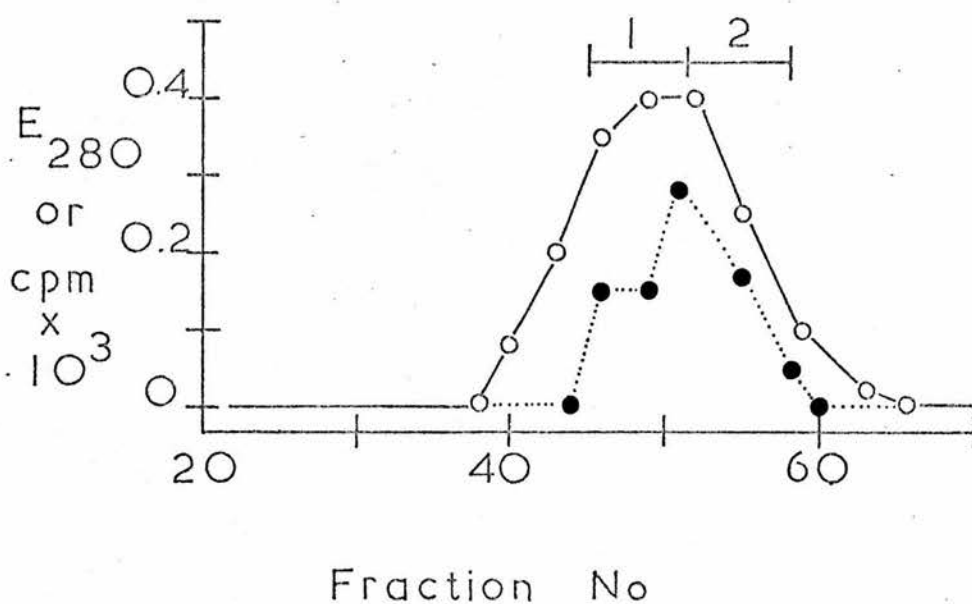


Fig. 47. Chromatography of the 5% acetic acid soluble material obtained from a large scale fission of inhibited pepsin C by cyanogen bromide on Sephadex G-50 (3 x 37 cm), equilibrated with 5% acetic acid.

—○— - E₂₈₀
●..... - radioactivity

12. Large scale degradation by cyanogen bromide.

Inhibited pepsin C (213 mg) was dissolved in 7 ml of 80% formic acid and 450 mg of cyanogen bromide in 1 ml of 80% formic acid was added. This mixture was incubated at room temperature for 24 hours, at which time 50 ml of water was added and the mixture was freeze-dried.

5% acetic acid (5 ml) was added to the lyophilised powder. The insoluble material was centrifuged off and washed with another 5 ml of 5% acetic acid. Samples of the supernatant and washings were assayed for radioactivity separately and from this it was found that 30% of the label had been extracted into the acetic acid. The supernatant and washings were applied to a column of Sephadex G-50 (3 x 37 cm), equilibrated with 5% acetic acid. Fractions (5 ml) were collected and 0.05 ml samples of each fraction were taken for radioactive assay (Fig. 47). A 100% recovery of radioactive material was achieved from the column. Fractions were pooled as indicated by the bars.

12.1 Further separation of CN1.

This was taken to dryness in vacuo and re-suspended in 2 ml of 5% acetic acid. The slight trace of insoluble material was centrifuged off and counting the supernatant revealed that all the radioactivity was in this fraction so the precipitate was discarded. Half of the supernatant was applied to No. 1 paper in a 4 inch strip and subjected to electrophoresis at pH 3.6 at 2 kV for 100 minutes. After drying, the paper was scanned (Fig. 48).

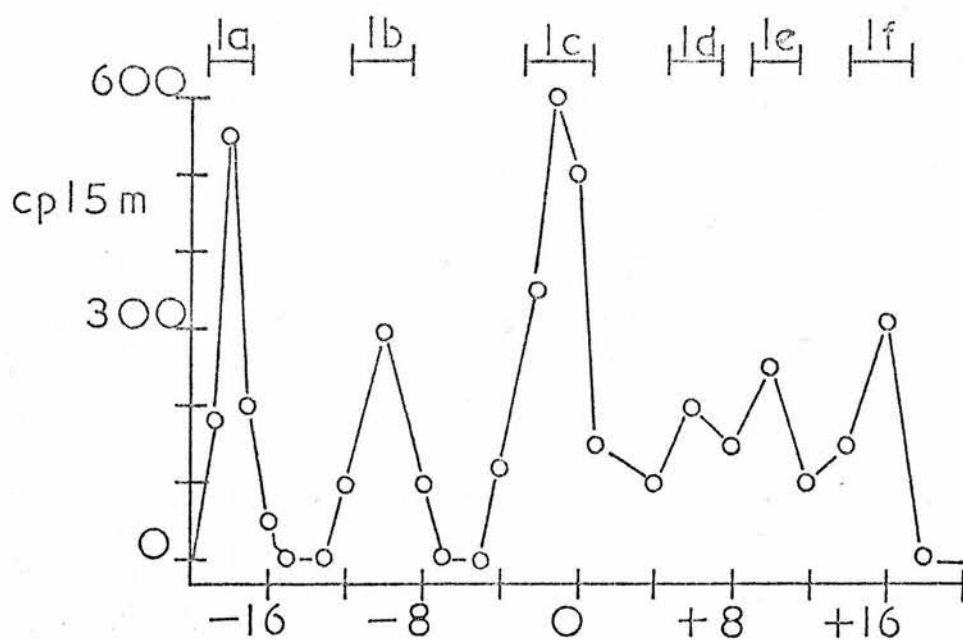


Fig. 48. Electrophoresis at pH 3.6 of fraction CN1

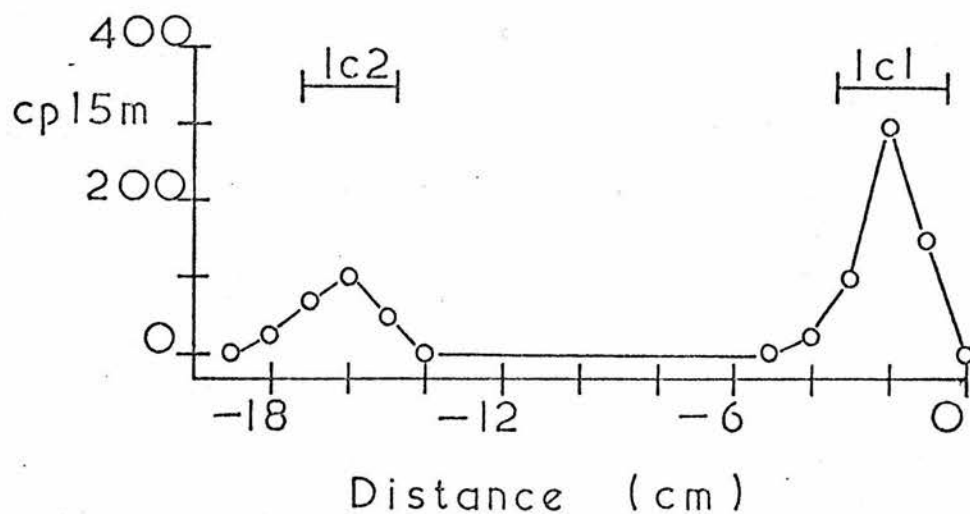


Fig. 49. Electrophoresis at pH 2.0 of CN1c, obtained from the pH 3.6 run.

Fraction CN1c was cut out, stitched into a fresh sheet and re-run at pH 2.0 at 2 kV for 2 hours (Fig. 49), giving CN1c1 and CN1c2.

Fractions CN1a, b, c1, c2, d, e and f were eluted from the paper with 5% acetic acid, hydrolysed for 24 hours and then heated at 105° for 1 hour in pH 6.5 buffer. The following amino acid analyses were obtained:-

	1a	1b	1c1	1c2	1d	1e	1f
Asp	-	0.5	0.7	16.0	7.3	1.3	-
Thr	-	1.1	1.0	5.1	2.0	0.2	-
Ser	2.8	2.0	1.3	7.2	3.4	0.5	-
Hsr	-	0.2	0.9	2.5	0.5	-	-
Glu	-	0.7	1.8	30.5	11.9	2.1	-
Pro	2.5	1.6	2.2	8.9	1.9	0.5	-
Gly	0.7	0.8	2.1	15.7	7.1	0.9	-
Ala	-	1.0	1.5	9.8	2.8	0.2	-
Val	3.7	1.2	0.9	9.6	2.1	-	-
Ile	1.6	0.6	0.4	8.8	2.2	-	-
Leu	3.9	1.2	1.3	19.0	3.2	-	-
Nle	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Tyr	0.6	-	-	1.7	0.5	-	-
Phe	-	-	0.3	4.7	1.2	-	-
Lys	8.0	1.7	-	-	-	-	-

Interpretation of these results is complicated.

Fraction 1a does not contain any aspartate or glutamate and DNM was used as an inhibitor with the specific aim of reaction with carboxyl groups. The fraction also apparently contains 8 lysine residues but since pepsin C

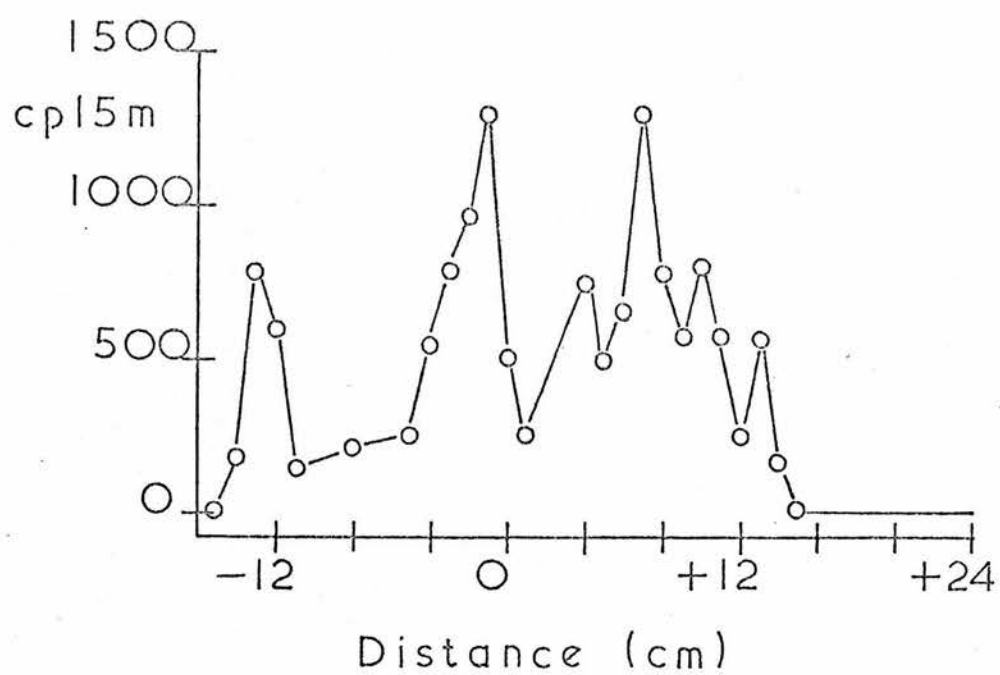


Fig. 50. Electrophoresis at pH 3.6 of fraction CN2.

contains only 4, this must represent heterogeneity of the fraction. There is no homoserine present either.

Fraction 1b also contains very little homoserine, has low values for the acidic residues but otherwise the composition would seem reasonable.

Fraction 1c1 has an acceptable composition but the yield was low.

Fractions 1c2 and 1d obviously contain a number of peptides.

Fraction 1e again has a lack of homoserine.

Fraction 1f, most surprisingly, contains only norleucine and migrates very similarly to the inhibitor breakdown products. From this it would appear that some of the inhibitor can be released from the enzyme, even under acid conditions.

These results present a very much more complicated picture than was obtained from the pilot experiment.

12.2 Further separation of CN2.

This material was dried in vacuo, re-dissolved in 5% acetic acid and applied to No. 1 paper in a 4 inch strip. The paper was subjected to electrophoresis at pH 3.6 at 2 kV for 100 minutes (Fig. 50). Further treatment of the labelled bands obtained from this by electrophoresis at pH 2.0 or by chromatography on Sephadex G-10 gave very low recoveries of material and amino acid analysis, N-terminal analysis and carboxypeptidase A digestion did not give any useful information whatsoever.

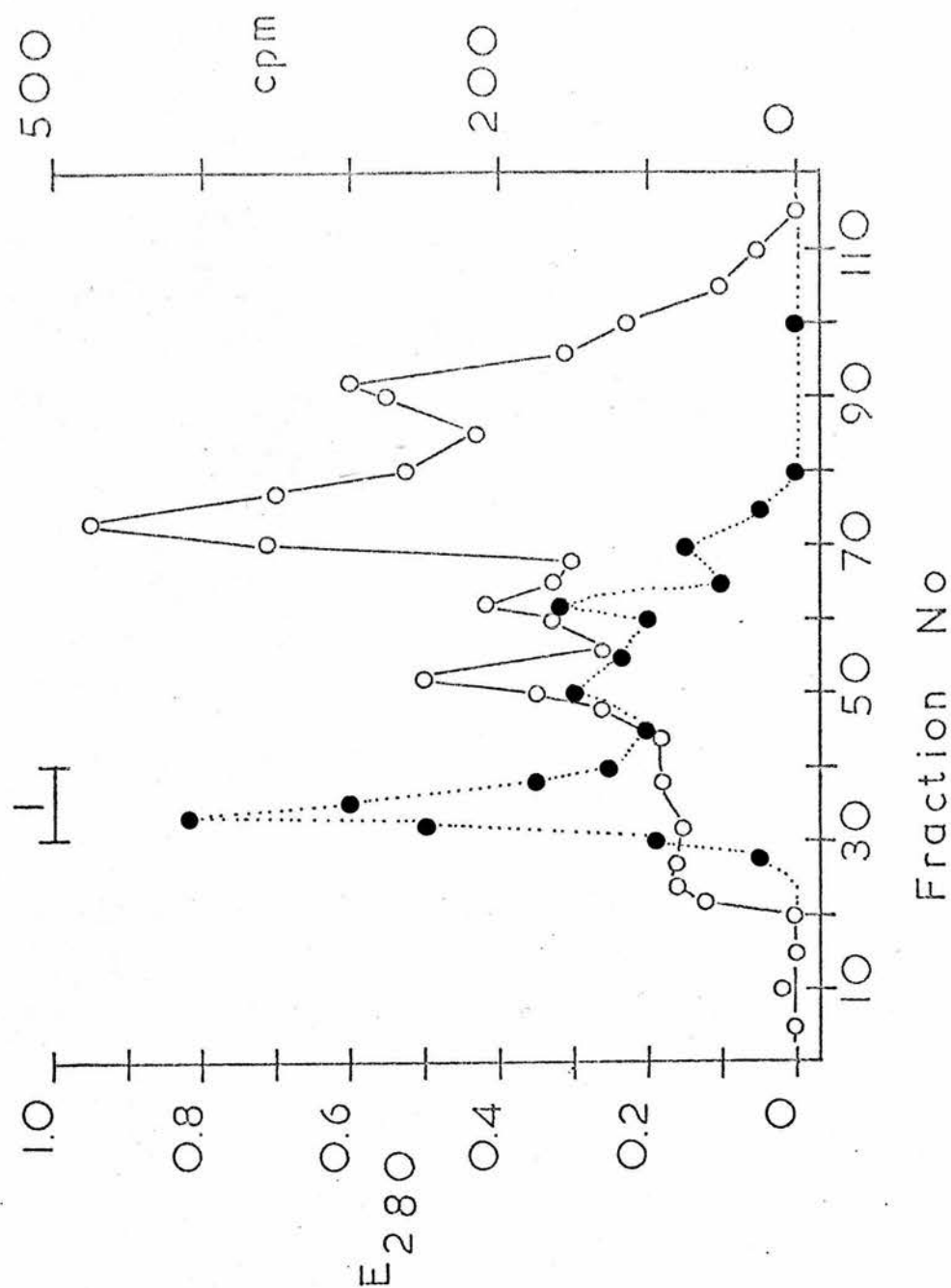


Fig. 51. Chromatography of the peptic digest of the material insoluble in 5% acetic acid obtained after cyanogen bromide fission, on Bio-Gel P 4 (3 x 45 cm), equilibrated with 5% acetic acid.

—○— - E₂₈₀
●..... - radioactivity

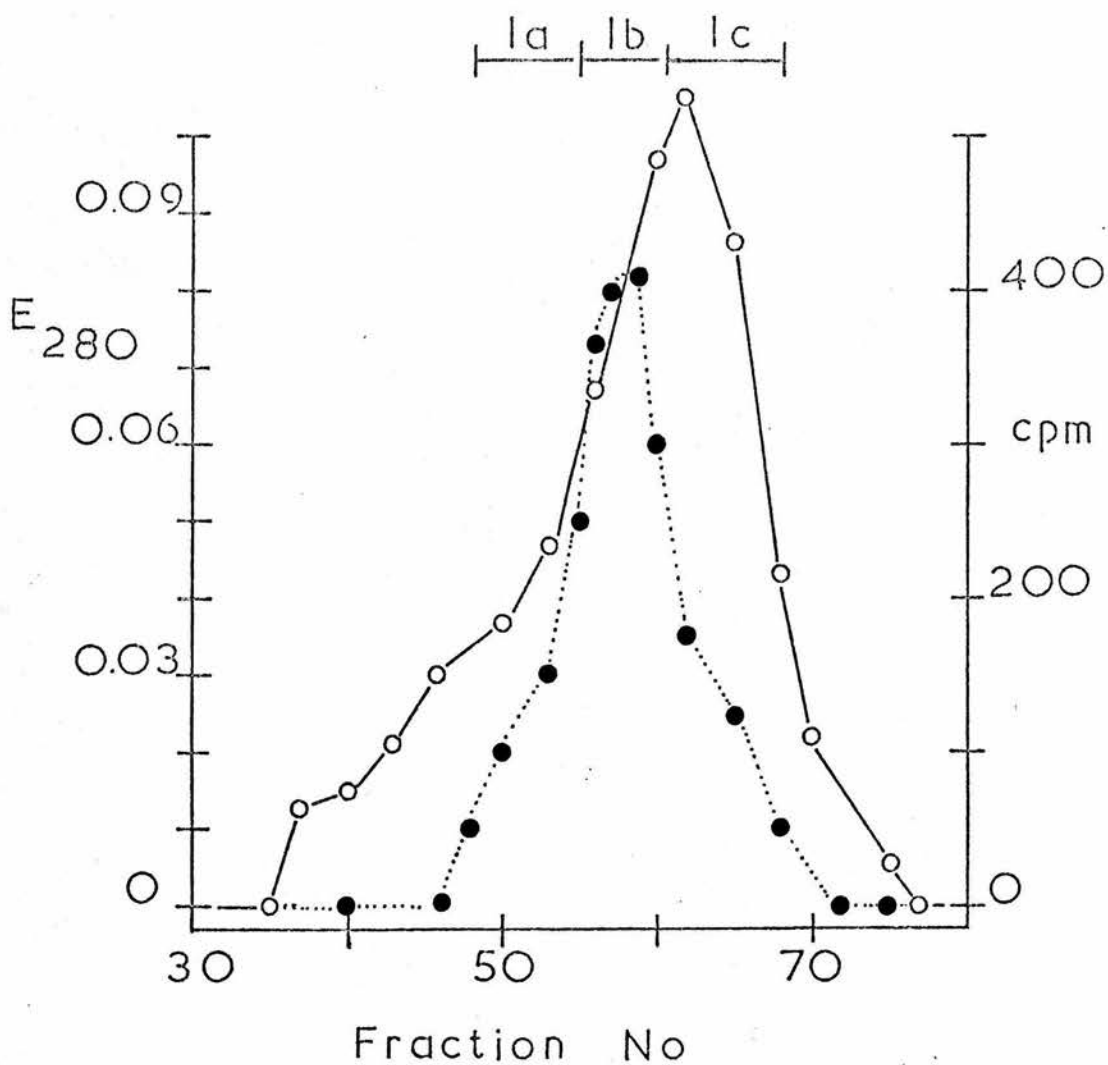


Fig. 52. Re-chromatography of fraction CNP1 on Bio-Gel P 6 (2.2 x 50 cm), equilibrated with 5% acetic acid.

—○— - E₂₈₀
●..... - radioactivity

Thus, the soluble fraction from the cyanogen bromide fission proved useless with regard to trying to isolate a labelled peptide but it did show that some of the inhibitor was capable of being split off the enzyme, even under acid conditions.

12.3 Peptic digestion of the insoluble material from the cyanogen bromide fission.

Pepsin (4 mg) was added to the insoluble material from the cyanogen bromide fission suspended in 10 ml of 0.02N-HCl. This mixture was shaken for 22 hours at 37° at which time the small amount of insoluble material remaining was centrifuged off and washed with 5% acetic acid. Samples of the supernatant and washings were assayed for radioactivity separately and these showed that 70% of the total radioactivity was in the supernatant fraction.

The supernatant and washings were applied to a column of Bio-Gel P 4 (3 x 45 cm), equilibrated with 5% acetic acid. Fractions (5 ml) were collected and 0.05 ml of each was taken for radioactive assay (Fig. 51). Fraction CNPl was pooled as shown and re-chromatographed on a column of Bio-Gel P 6 (2.2 x 50 cm), equilibrated with 5% acetic acid. Fractions (2 ml) were collected and 0.2 ml samples were used for scintillation counting (Fig. 52). A 90% recovery of radioactive material was achieved from this column. Fractions CNPla, b and c were pooled as shown.

a) Further separation of CNPla.

This was dried in vacuo, re-dissolved in 0.5 ml of 5% acetic acid and applied to No. 1 paper in a 3 inch strip.

The paper was subjected to electrophoresis at pH 3.6 at 2 kV for 2 hours (Fig. 53). Band 1a1 was cut out, stitched into a fresh sheet and re-run at pH 2.0 (Fig. 54). The radioactive band from this was eluted with 5% acetic acid and a sample was hydrolysed for 24 hours.

Amino acid analysis gave the composition, relative to norleucine = 1.0:-

	Asp	Thr	Ser	Pro	Gly	Ala	Val	Ile	Leu	Nle
1a1	1.1	2.6	1.9	0.6	2.0	1.7	0.8	0.9	1.4	1.0

N-terminal analysis by DNS-Cl gave DNS-alanine as the major spot with a minor spot of DNS-isoleucine.

The remainder of the material was treated with carboxypeptidase A and samples were taken for analysis at 0.5, 1 and 4 hours. The following amino acids (in μ moles/ μ mole of CNPla) were released:-

	Thr	Ser	Ala	Leu
Hours				
0.5	0.05	0.07	0.15	0.77
1.0	0.07	0.09	0.20	0.76
4.0	0.11	0.12	0.20	0.80

It was impossible to deduce much sequence information from these results at the time but, with the information which was obtained in the experiments shortly to be described, it is possible to make an attempt to rationalise them.

If, for every μ mole of labelled material, 0.8 μ moles is contributed by a peptide with N-terminal alanine and the remaining 0.2 μ moles by a peptide with N-terminal isoleucine,

then the following scheme can be proposed:-

I) Ala-(Gly,Thr,Ser,Leu,Ile,Val,Asp,Thr,Gly,Thr,Ser)-Pro-Ala-Leu
 II) Ile-(Val,Asp,Thr,Gly)-Thr-Ser

Treatment of this mixture of peptides with carboxypeptidase A should release (from peptide I) leucine very rapidly and perhaps a small amount of alanine, since carboxypeptidase A does not readily break -Pro-X bonds (Ambler 1967). From peptide II serine should be released fairly slowly and the release of threonine should parallel this. Glycine is not very readily released by carboxypeptidase so the hydrolysis might stop at this point. This explains the observed carboxypeptidase results and, if one allows 0.8 residues for each amino acid in peptide I and 0.2 residues for each in peptide II, the following amino acid composition is obtained:-

Asp	Thr	Ser	Pro	Gly	Ala	Val	Ile	Leu	Nle
1.0	2.6	1.8	0.8	1.8	1.6	1.0	1.0	1.6	1.0

which agrees fairly well with the observed composition. It is perhaps a little more difficult to see how these two peptides should behave similarly on gel-filtration and electrophoresis. Since they both contain only neutral amino acids (the aspartate is presumably modified by the neutral inhibitor) this might explain the similarities in mobility. Fig. 54 does show some evidence of tailing. This proposal of two peptides is not, of course, the only explanation for the results from this fraction.

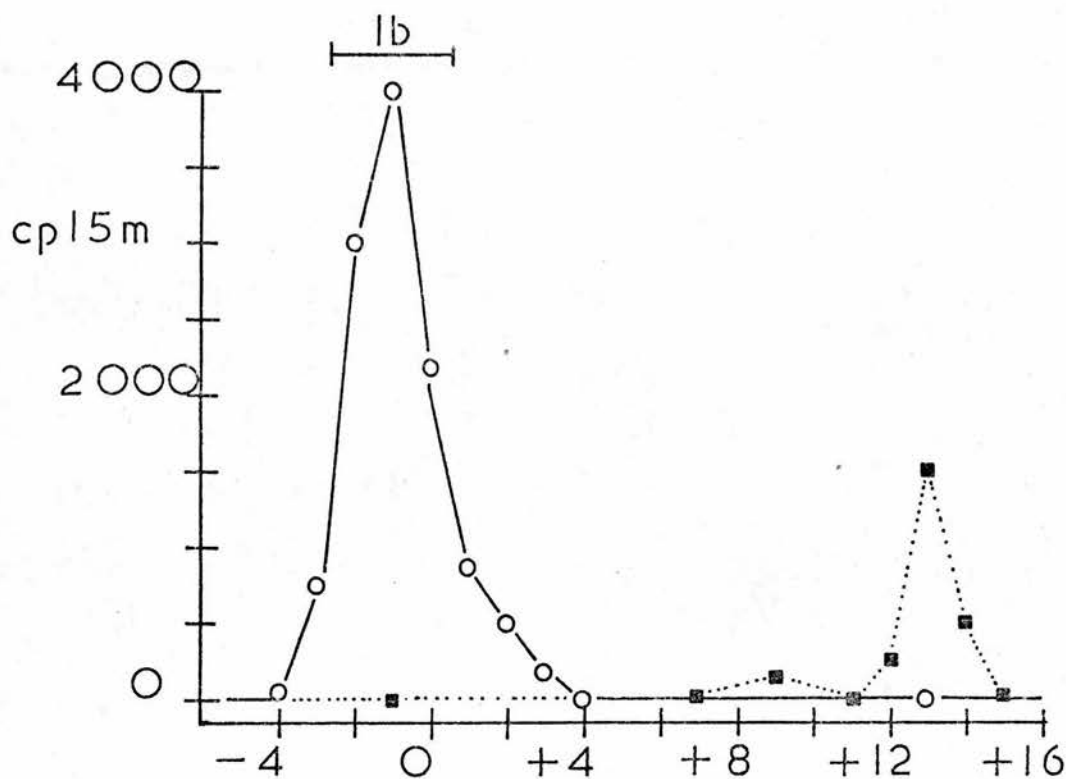


Fig. 55. Electrophoresis at pH 3.6 of fraction CNPlb

—○— - Fraction CNPlb
■..... - Inhibitor breakdown products

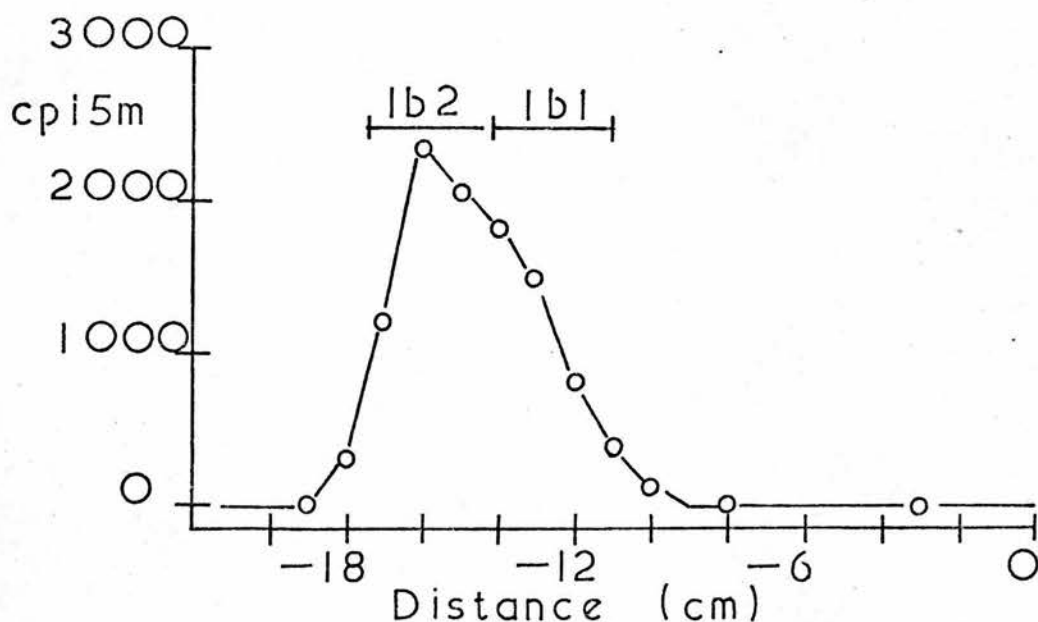


Fig. 56. Electrophoresis at pH 2.0 of CNPlb obtained from the pH 3.6 run.

b) Further separation of CNPlb.

This was dried in vacuo, re-dissolved in 0.5 ml of 5% acetic acid and applied to No. 1 paper in a 3 inch strip with inhibitor breakdown products as marker. The paper was subjected to electrophoresis at pH 3.6 (Fig. 55). Band lb was cut out, stitched into a fresh sheet and re-run at pH 2.0 (Fig. 56). Two bands, lb1 and lb2, as indicated by the bars, were eluted from this with 5% acetic acid. Amino acid analysis (after hydrolysis for 24 hours) gave the following compositions:-

	Asp	Thr	Ser	Hsr	Glu	Pro	Gly	Ala	Val	Ile	Leu	Nle	Phe
lb1	3.4	3.5	4.4	0.1	3.9	4.1	4.8	2.2	2.5	3.6	3.4	1.0	1.5
lb2	1.6	3.0	3.3	0.2	2.6	5.0	2.6	3.2	2.6	1.5	2.7	1.0	1.6
lb2a	1.6	3.0	3.1	0.2	2.0	5.0	2.5	2.9	2.3	1.2	2.3	1.0	1.4
(see text)													

N-terminal analysis by DNS-Cl revealed that both fractions had DNS-leucine, DNS-alanine and DNS-glycine, indicating them to be heterogeneous. An attempt was made to further purify lb2 (being the smaller of the two fractions) by chromatography on Bio-Gel P 2 and pooling the resulting fractions containing the radioactive label. After hydrolysis for 24 hours the amino acid analysis of lb2a (see above) was virtually the same as before chromatography. Since both lb1 and lb2a were obviously heterogeneous, nothing further was done with them.

c) Further separation of CNPlc.

This was dried in vacuo, re-dissolved in 0.5 ml of 5% acetic acid, applied to No. 1 paper in a 3 inch strip and subjected to electrophoresis at pH 3.6 (Fig. 57). Band lc

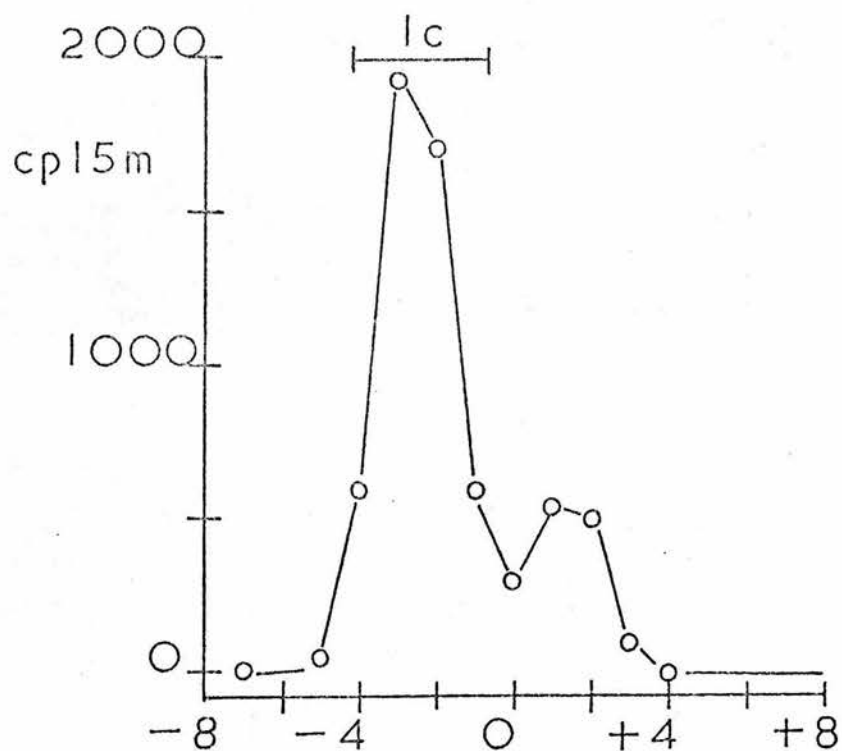


Fig. 57. Electrophoresis at pH 3.6 of fraction CNPlc.

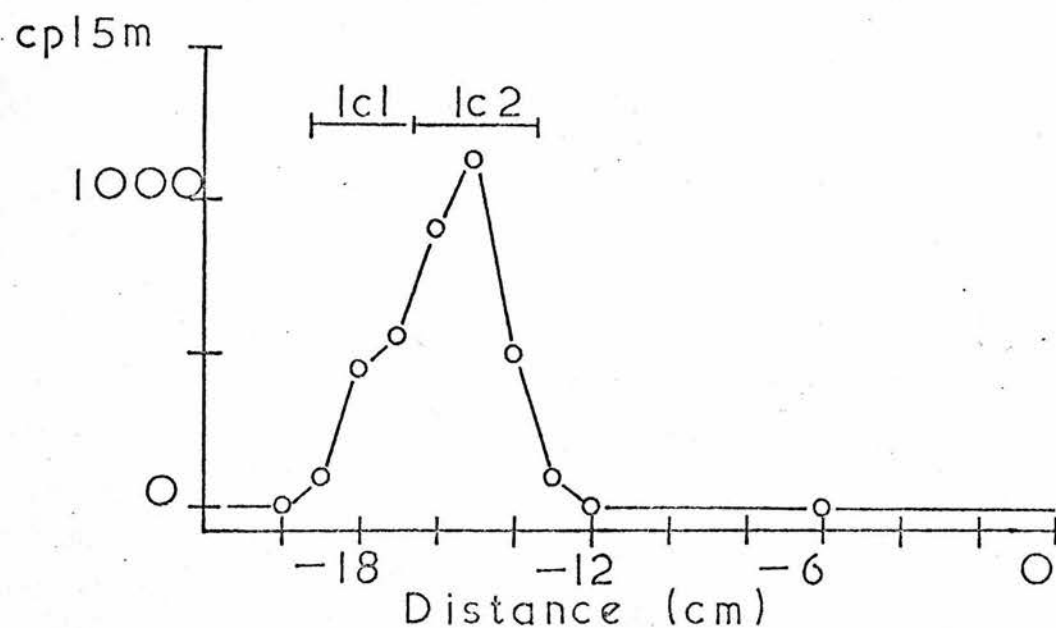


Fig. 58. Electrophoresis at pH 2.0 of CNPlc obtained from the pH 3.6 run.

was cut out, stitched into a fresh sheet and re-run at pH 2.0 (Fig. 58). This gave fractions 1c1 and 1c2. These were eluted with 5% acetic acid and after hydrolysing samples for 24 hours, amino acid analysis gave the following results, relative to norleucine = 1.0:-

	Asp	Thr	Ser	Hsr	Glu	Pro	Gly	Ala	Val	Ile	Leu	Nle	Tyr	Phe
1c1	3.4	8.1	4.0	0.6	7.8	9.0	5.5	10.0	6.6	1.5	6.5	1.0	-	1.8
1c2	6.0	6.4	5.8	0.1	8.0	11.5	6.7	3.2	4.4	5.7	4.8	1.0	0.3	4.5

N-terminal analysis by DNS-Cl showed DNS-alanine, DNS-leucine and DNS-proline for 1c1 and DNS-alanine, DNS-leucine and DNS-glycine for 1c2. These fractions were obviously heterogeneous and it was not thought worthwhile to try to separate them further because of the high level of (presumably) non-labelled peptides contaminating the labelled one.

12.4 Conclusions from the cyanogen bromide fission.

As a general comment on the cyanogen bromide fission, it did not prove at all helpful simply by cleaving with cyanogen bromide alone. Peptic digestion of the insoluble material from the cyanogen bromide fission gave some information which, although difficult to interpret on its own merit, may be supplemented by information gained from a straightforward peptic digest.

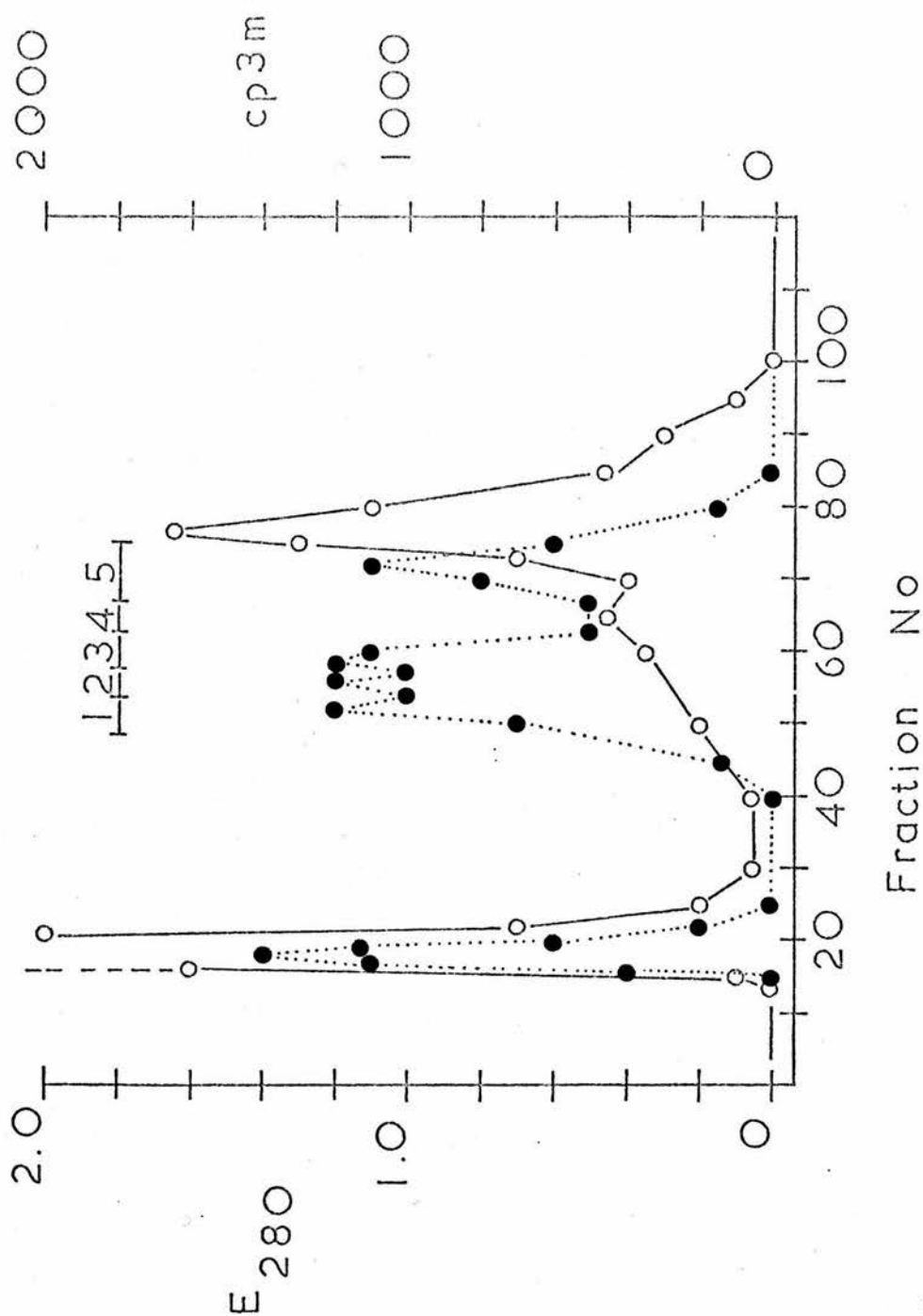


Fig. 59. Chromatography of the peptic digest of inhibited pepsin C on Bio-Gel P 6 (3.2 x 50 cm), equilibrated with 5% acetic acid.

—○— - E₂₈₀
●..... - radioactivity

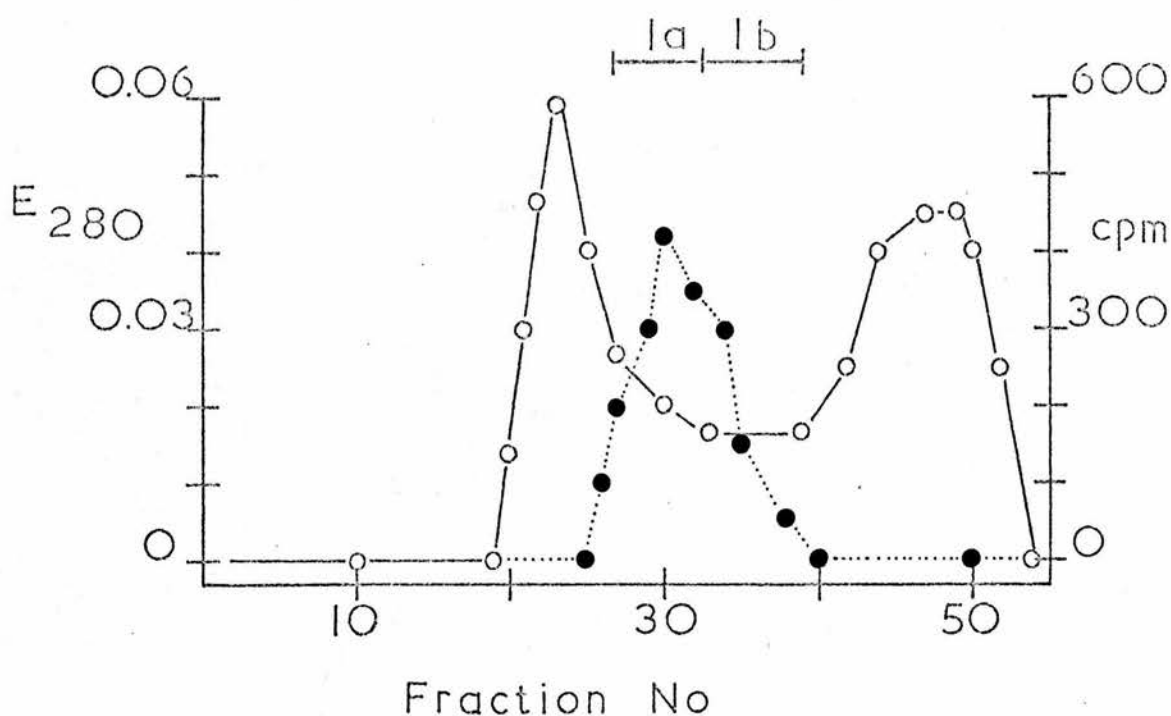


Fig. 60. Re-chromatography of fraction P1 on Bio-Gel P 2 (2 x 50 cm), equilibrated with 5% acetic acid.

—○— - E_{280}
●..... - radioactivity

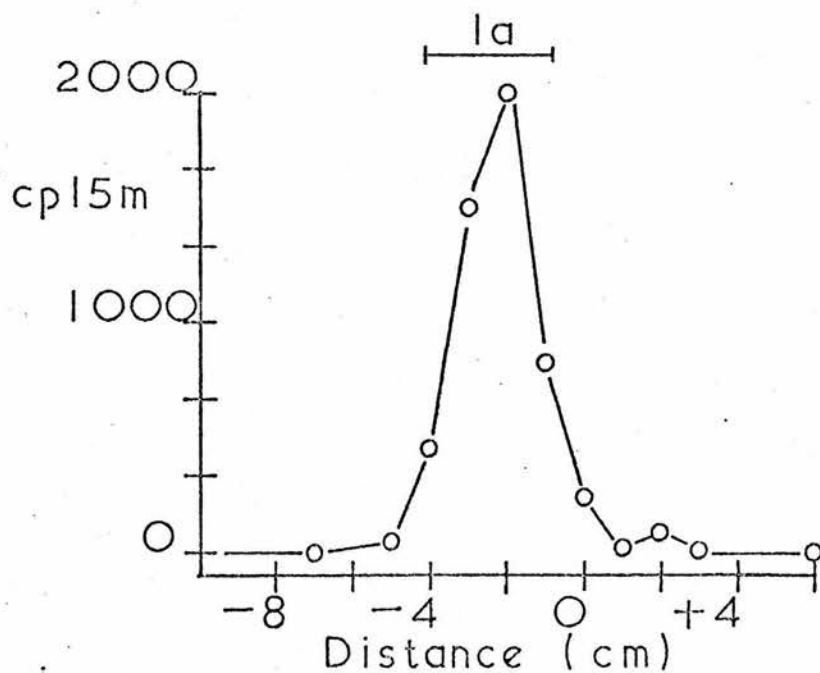


Fig. 61. Electrophoresis of fraction Pla at pH 3.6.

13. Large scale degradation of inhibited pepsin C by pepsin.

Inhibited pepsin C (175 mg) was suspended in 10 ml of 0.02N-HCl and pepsin (7 mg) was added. This mixture was shaken at 37° for 23 hours, at which time the insoluble material remaining was centrifuged off. The supernatant was assayed for radioactivity and 80% of the total radioactivity was found in this fraction.

The supernatant was applied to a column of Bio-Gel P 6 (3.2 x 50 cm), equilibrated with 5% acetic acid. Fractions (5 ml) were collected and samples were taken for radioactive assay. The elution profile is shown in Fig. 59. The material which emerged at the void volume of the column was presumably undigested pepsin C and this was freeze-dried and stored in the deep-freeze.

Fractions were pooled as follows:-

49-53	designated	P1
54-57	"	P2
58-62	"	P3
63-66	"	P4
67-72	"	P5

13.1 Further separation of P1.

This was dried in vacuo, re-dissolved in 5% acetic acid and applied to a column of Bio-Gel P 2 (2 x 50 cm), equilibrated with 5% acetic acid. Fractions (2 ml) were collected and 0.1 ml samples were taken for radioactive assay (Fig. 60). Fractions P1a and P1b were pooled as indicated. Samples of these were taken for amino acid analysis:-

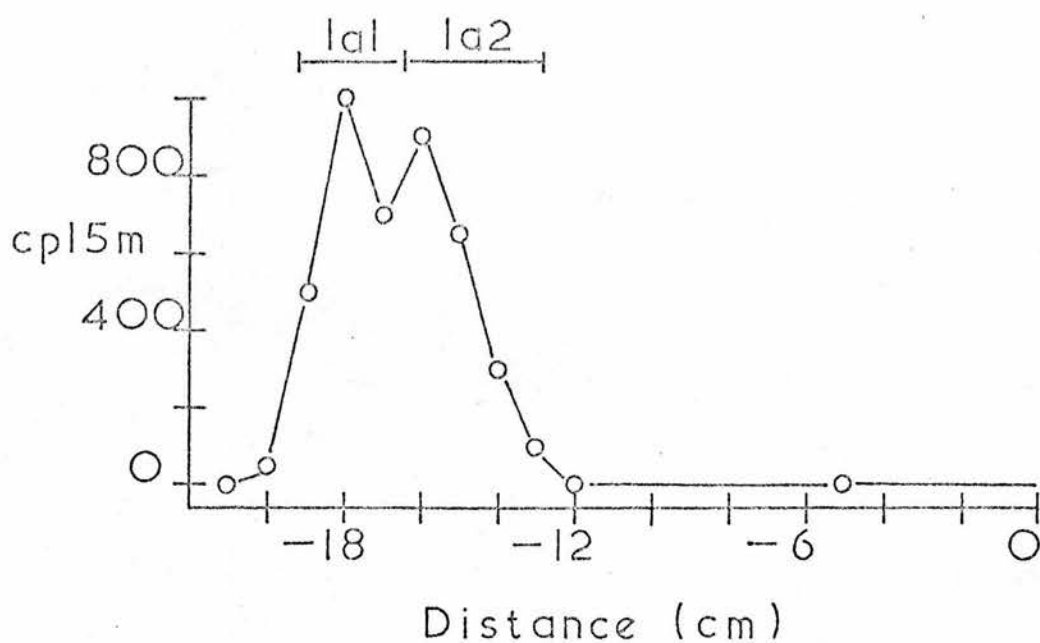


Fig. 62. Electrophoresis at pH 2.0 of Pla obtained from the pH 3.6 run.

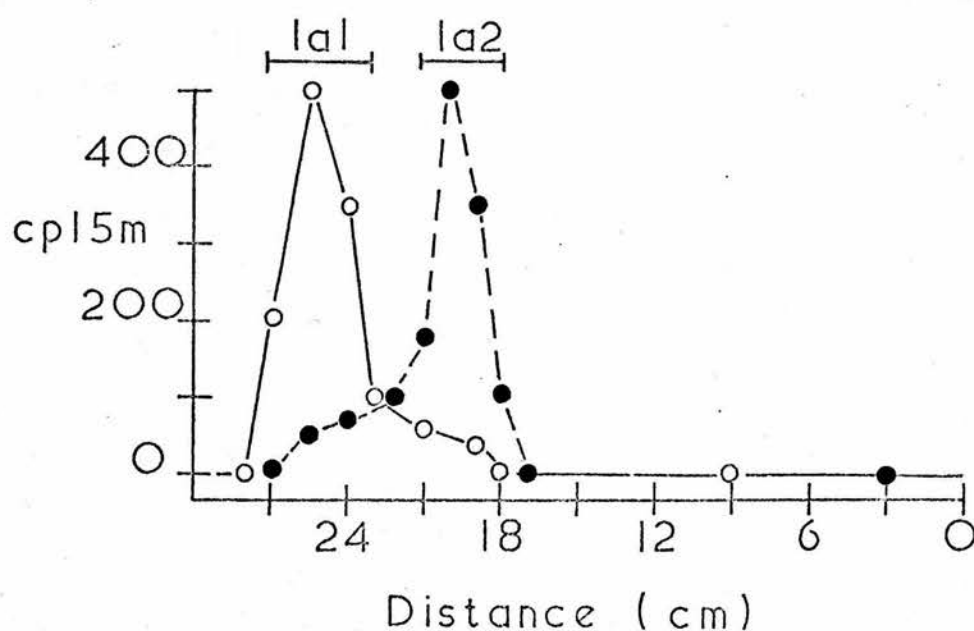


Fig. 63. Chromatography in t-butanol:methyl ethyl ketone: water::2:2:1 of fractions Plal and la2, obtained from the electrophoresis at pH 2.0

—○— - Fraction Plal
 -●- - Fraction Pla2

	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Nle	Phe
Pla	4.3	7.7	7.2	7.7	8.5	6.2	9.0	3.4	0.6	3.4	4.1	1.0	1.6
Plb	2.1	3.4	2.9	3.9	1.8	2.9	3.3	2.4	-	1.0	2.8	1.0	0.3

Further separation was obviously required but, again, tyrosine was significant by its absence.

a) Separation of Pla.

This was dried in vacuo and re-suspended in 0.5 ml of 5% acetic acid. The insoluble material was centrifuged off and washed with a further 0.5 ml of 5% acetic acid. Assay of the supernatant and washings showed that all the radioactivity was in these fractions and the precipitate was discarded.

The soluble fractions were applied to No. 1 paper in a 3 inch strip and subjected to electrophoresis at pH 3.6 (Fig. 61). The radioactive band from this was cut out, stitched into a fresh sheet and re-run at pH 2.0. This gave two partially separated bands (Fig. 62). These were cut out separately and the material was concentrated on the paper by folding the paper along its length into a kind of tent and allowing 5% acetic acid to diffuse up to the apex. The papers were then dried, stitched into fresh sheets and run in t-butanol:methyl ethyl ketone:water::2:2:1 overnight. Scanning the papers after drying (Fig. 63) showed that 1a1 had migrated with an R_F of 0.53 whereas 1a2 had an R_F of 0.36. These were eluted from the paper with 5% acetic acid and samples were hydrolysed for 24 hours for amino acid analysis:-

	Asp	Thr	Ser	Gly	Val	Ile	Nle
1a1	1.0	1.0	-	-	0.7	0.6	1.0
1a2	1.0	1.8	1.0	1.1	0.7	0.6	1.0

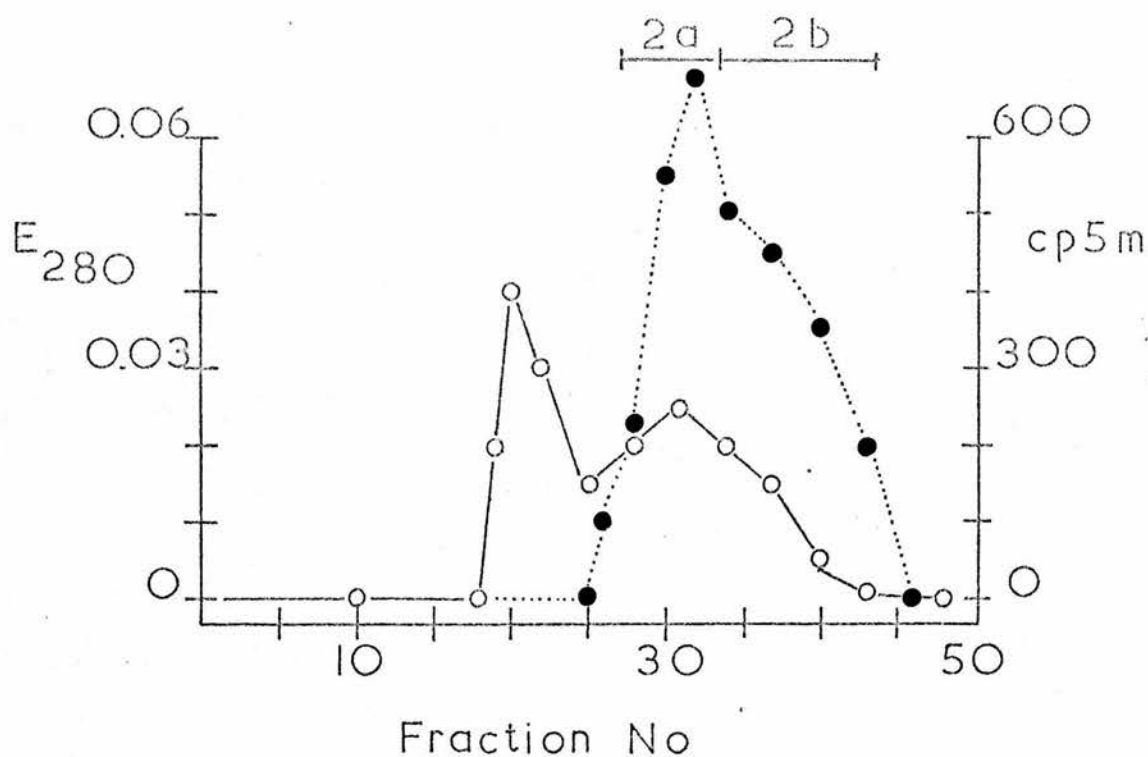


Fig. 64. Re-chromatography of fraction P2 on Bio-Gel P 2 (2 x 50 cm), equilibrated with 5% acetic acid.

—○— - E_{280}
●..... - radioactivity

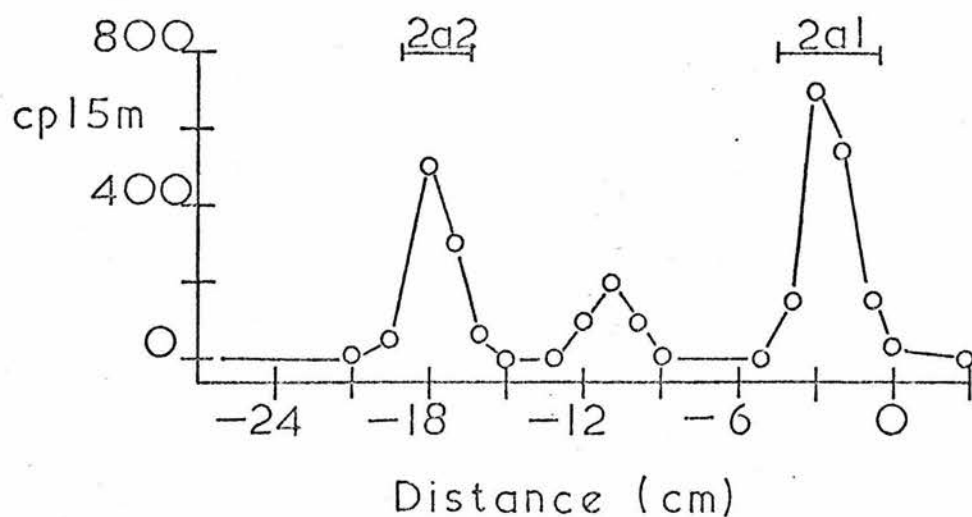


Fig. 65. Electrophoresis at pH 3.6 of fraction P2a.

N-terminal analysis by DNS-Cl showed that both fractions had DNS-isoleucine. Degradation of 1a1 with aminopeptidase released isoleucine and valine very quickly but no free threonine was found. Since threonine is usually released very rapidly by aminopeptidase (Light 1967) whereas aspartate is not released, this suggests the sequence of this peptide to be:-

Ile-Val-Asp-Thr

although it must be remembered that the aspartate residue is probably modified by an inhibitor residue and this may affect the digestion.

Treatment of 1a2 with carboxypeptidase A released only serine very, very slowly. Since the peptide had N-terminal isoleucine, an overlap with 1a1 suggests that the sequence may be:-

Ile-Val-Asp-Thr-(Gly,Thr)-Ser

b) Separation of Plb.

Unfortunately this material was lost in an accident in the laboratory.

13.2 Further separation of P2.

This was dried in vacuo and re-dissolved in 1 ml of 5% acetic acid. This solution was applied to a column of Bio-Gel P 2 (2 x 50 cm), equilibrated with 5% acetic acid. Fractions (2 ml) were collected and 0.1 ml samples were taken for scintillation counting (Fig. 64). Fractions P2a and P2b were pooled as indicated and samples of these were hydrolysed for 24 hours for amino acid analysis:-

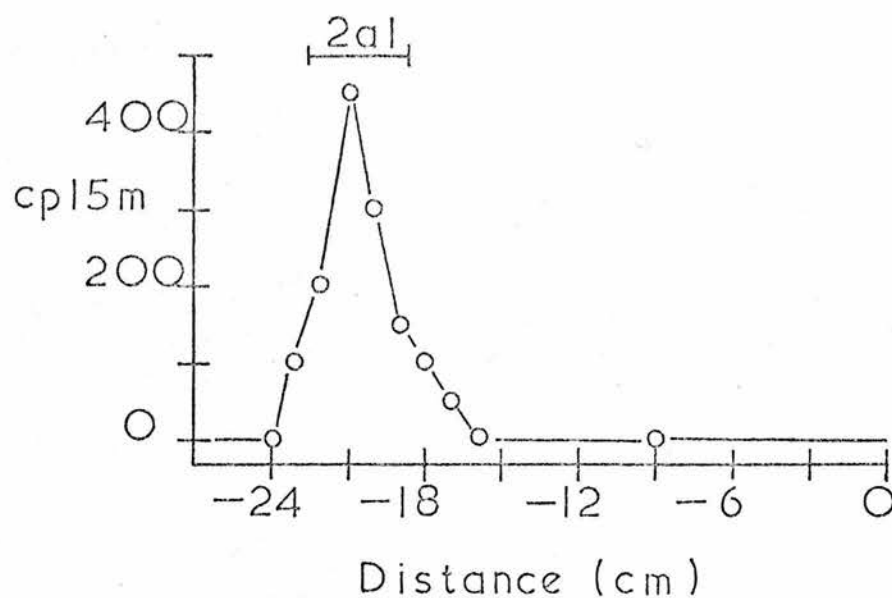


Fig. 66. Electrophoresis at pH 2.0 of P2a1 obtained from the pH 3.6 run.

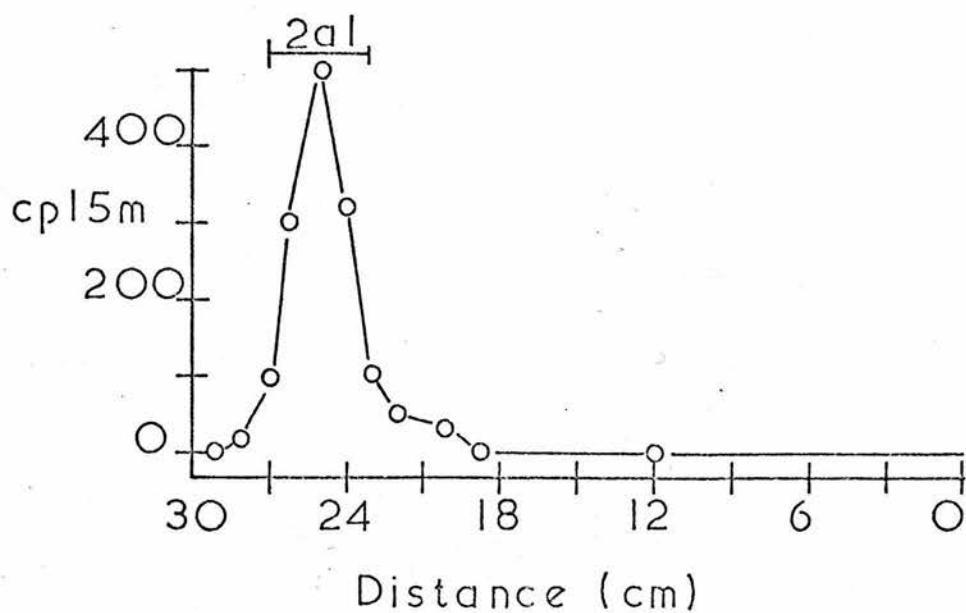


Fig. 67. Chromatography in t-butanol:methyl ethyl ketone: water::2:2:1 of P2a1 obtained from the electrophoresis at pH 2.0.

	Asp	Thr	Ser	Glu	Pro	Gly	Ala	$\frac{1}{2}$ Cys	Val	Ile	Leu	Nle	Tyr	Phe
2a	5.0	7.0	7.0	11.0	5.0	6.8	6.8	0.7	5.6	2.8	5.8	1.0	0.5	0.5
2b	2.4	3.6	4.1	2.7	1.3	5.0	2.5	1.4	3.2	2.5	6.8	1.0	0.6	0.2

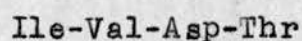
a) Separation of P2a.

This was dried in vacuo, re-dissolved in 5% acetic acid and applied to No. 1 paper in a 3 inch strip. The paper was subjected to electrophoresis at pH 3.6 and this gave the scan shown in Fig. 65. Band 2a1 was cut out, stitched into a fresh sheet and re-run at pH 2.0 (Fig. 66). The radioactive band from this was cut out, concentrated for paper chromatography as described previously, and run in butanol: methyl ethyl ketone:water for 15 hours. The radioactive material migrated with an R_F of 0.54 (Fig. 67).

This band was eluted from the paper with 5% acetic acid, as was band 2a2, and samples were hydrolysed for 24 hours for analysis. The compositions were as follows:-

	Asp	Thr	Gly	Val	Ile	Nle
2a1	1.1	1.0	-	1.0	0.8	1.0
2a2	-	-	0.5	-	-	1.0

N-terminal analysis of 2a1 by DNS-Cl gave DNS-isoleucine. Treatment of 2a1 with carboxypeptidase A liberated only threonine. Therefore this peptide is probably:-



Band 2a2 contained norleucine and a fractional amount of glycine and migrated as if it had at least a partial positive charge. It is rather difficult to see how DNM could attack a glycine residue in pepsin C so this fraction

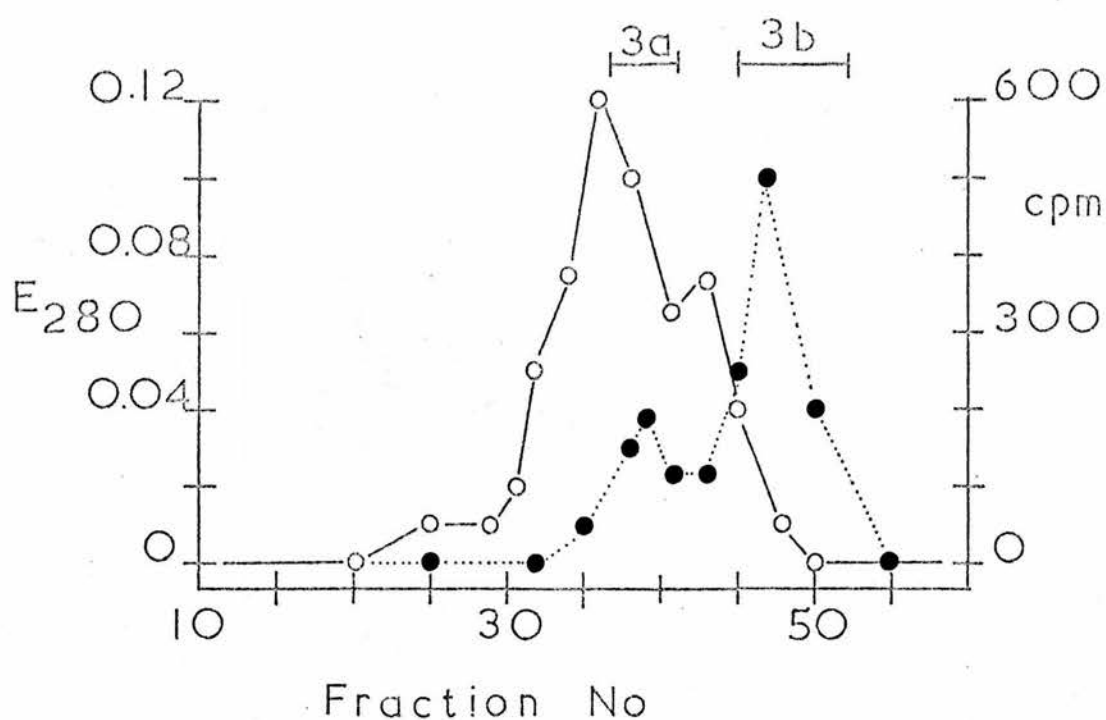


Fig. 68. Re-chromatography of fraction P3 on Bio-Gel P 2 (2 x 50 cm), equilibrated with 5% acetic acid.

—○— - E_{280}
●..... - radioactivity

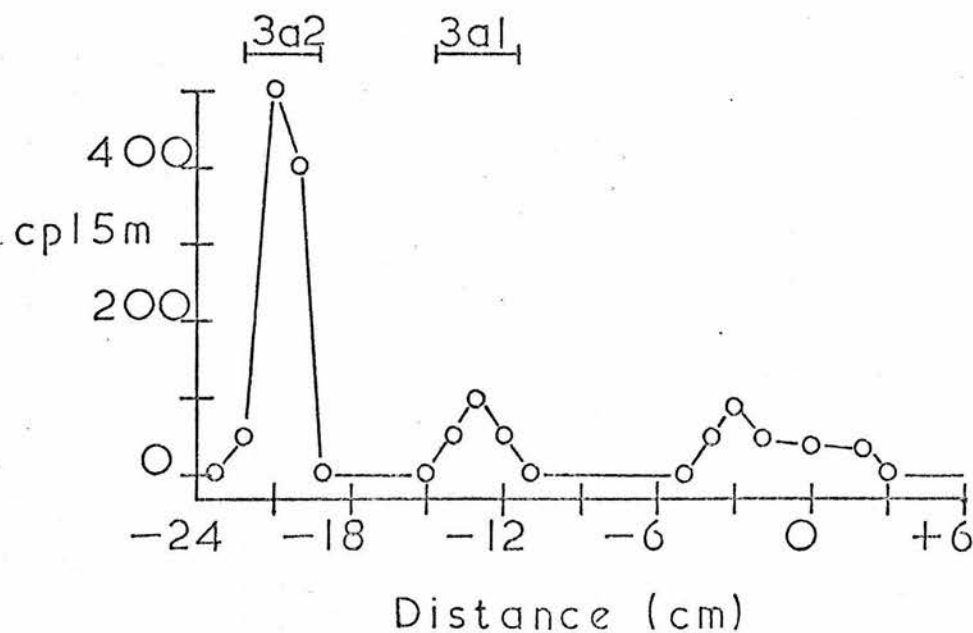


Fig. 69. Electrophoresis at pH 3.6 of fraction P3a.

may represent another breakdown product formed under acid conditions from the liberation of one of the inhibitor residues from the enzyme.

b) Separation of P2b.

This gave only one radioactive band on electrophoresis at pH 3.6, corresponding to band 2a2. Amino acid analysis showed only norleucine and so this fraction was discarded.

13.3 Separation of P3.

This was dried in vacuo and re-suspended in 1.5 ml of 5% acetic acid. The slight trace of insoluble material was centrifuged off and washed with 5% acetic acid. Radioactive assay showed that all the radioactivity was in the soluble fraction. This was applied to a column of Bio-Gel P 2 (2 x 50 cm), equilibrated with 5% acetic acid. Fractions (2 ml) were collected and samples were taken for scintillation counting (Fig. 68). Fractions P3a and P3b were pooled as shown and samples of each were hydrolysed for 24 hours for amino acid analysis:-

	Asp	Thr	Ser	Glu	Pro	Gly	Ala	$\frac{1}{2}$ Cys	Val	Met	Ile	Leu	Nle	Tyr	Phe
3a	8.2	6.5	10.7	10.5	3.9	13.6	5.2	0.7	8.0	-	6.1	13.5	1.0	1.5	1.1
3b	4.4	1.8	3.6	4.0	0.9	5.0	1.8	-	2.1	0.4	2.2	12.3	1.0	-	0.8

a) Separation of P3a.

This was dried in vacuo, re-dissolved in 5% acetic acid, applied to No. 1 paper in a 3 inch strip and subjected to electrophoresis at pH 3.6 (Fig. 69). This was also found to contain this mysterious band migrating towards the cathode (3a2).

Band 3a1 was eluted with 5% acetic acid and hydrolysed for 24 hours. Amino acid analysis showed the presence of large

amounts of the other amino acids in relation to norleucine and since the amount of labelled material present was so small this fraction was discarded.

b) Separation of P3b.

This fraction was lost in the same accident as fraction Plb.

13.4 Separation of P4.

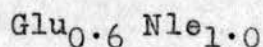
This was dried in vacuo and re-dissolved in 1 ml of 5% acetic acid. The solution was applied to a column of Bio-Gel P 2 (2 x 50 cm), equilibrated with 5% acetic acid. Fractions (2 ml) were collected and 0.1 ml samples were taken for radioactive assay (Fig. 70). Fractions P4a, b and c were pooled as shown and samples of each were hydrolysed for 24 hours for amino acid analysis:-

	Asp	Ser	Glu	Gly	Leu	Nle	Phe
4a	1.4	1.1	3.0	1.8	0.8	1.0	34.0
4b	0.3	0.5	1.1	0.9	0.4	1.0	-
4c	0.5	0.3	0.9	0.4	-	1.0	-

Fraction 4a must contain free phenylalanine.

a) Separation of 4a.

This was dried in vacuo, re-dissolved in 5% acetic acid, applied to No. 1 paper in a 2 inch strip with inhibitor breakdown products as marker and subjected to electrophoresis at pH 3.6 (Fig. 71). Band 4a1 was eluted with 5% acetic acid and, after hydrolysis for 24 hours, amino acid analysis showed it to have the composition:-



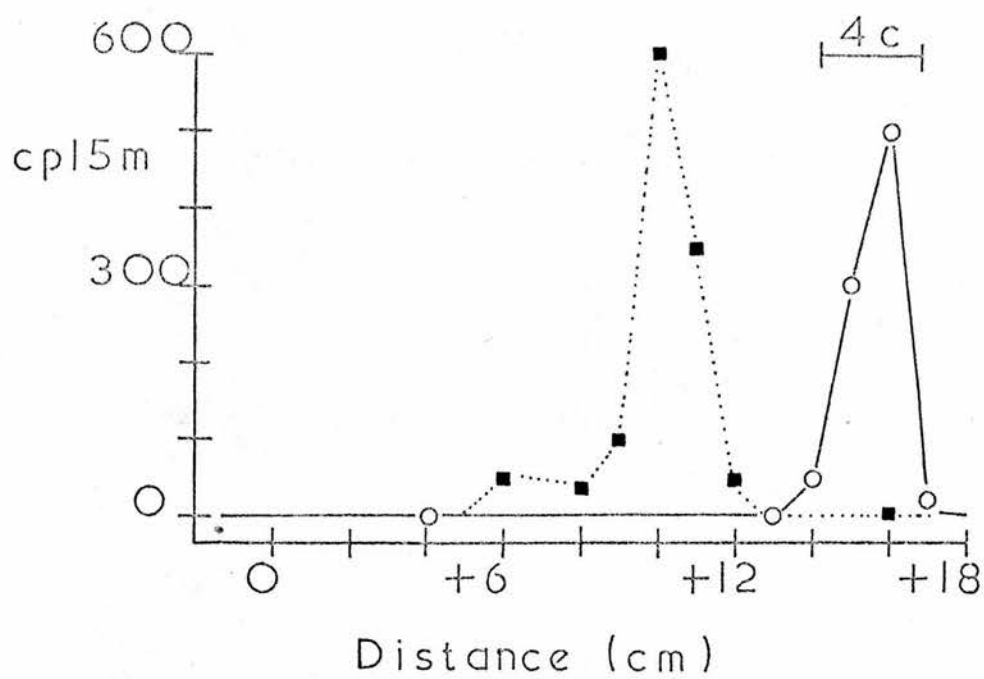


Fig. 72. Electrophoresis at pH 3.6 of fraction P_{4c}.

—○—

- Fraction P_{4c}

.....■.....

- Inhibitor breakdown products

This is probably the same as the material discussed in Section 10.4.

Fraction 4a2 was shown by amino acid analysis to be a complex mixture and it did not contain enough labelled material to make further separation worthwhile.

b) Separation of 4b.

This migrated identically with 4a1 and was not used for any further investigations.

c) Separation of 4c.

This was applied in a 2 inch strip to No. 1 paper with inhibitor breakdown products as marker and subjected to electrophoresis at pH 3.6 (Fig. 72). The radioactive material ran faster than the inhibitor breakdown products peak and after elution and hydrolysis amino acid analysis showed it to contain only norleucine. This also must be derived from an inhibitor molecule being split off the protein under acid conditions.

13.5 Separation of P5.

This was chromatographed on Bio-Gel P 2 as described for the other fractions. The radioactive fractions from this were pooled and subjected to electrophoresis at pH 3.6. This showed P5 to contain two radioactive bands, one migrating with the inhibitor breakdown products peak and one migrating identically with fraction P4c.

Both fractions were treated on No. 1 paper with triethylamine by allowing this to diffuse in from the edges of the band as in wetting a paper for electrophoresis. The papers were then re-run at pH 3.6. The mobility of the

radioactive material had not been altered. This suggests, since triethylamine was found to break the ester bond between an aspartate residue in pepsin and diazoacetyl phenylalanine methyl ester (Bayliss & Knowles 1968), that these fractions were genuine inhibitor breakdown products and the norleucine was not attached to perhaps the glutamate, usually found in the band which migrated with the inhibitor breakdown products.

13.6 Conclusions from the peptic digest.

It was only possible to isolate from the peptic digest of this batch of 3:1 inhibited enzyme, the peptides:-

Ile-Val-Asp-Thr

and

Ile-(Val,Asp,Thr,Gly,Thr)-Ser

This presumably means that one of the inhibitor residues, bound in the sequence:- Ile-Val-Asp-Thr, remains firmly attached to the enzyme under acid conditions whereas the other two residues are split off forming the various norleucine derivatives found.

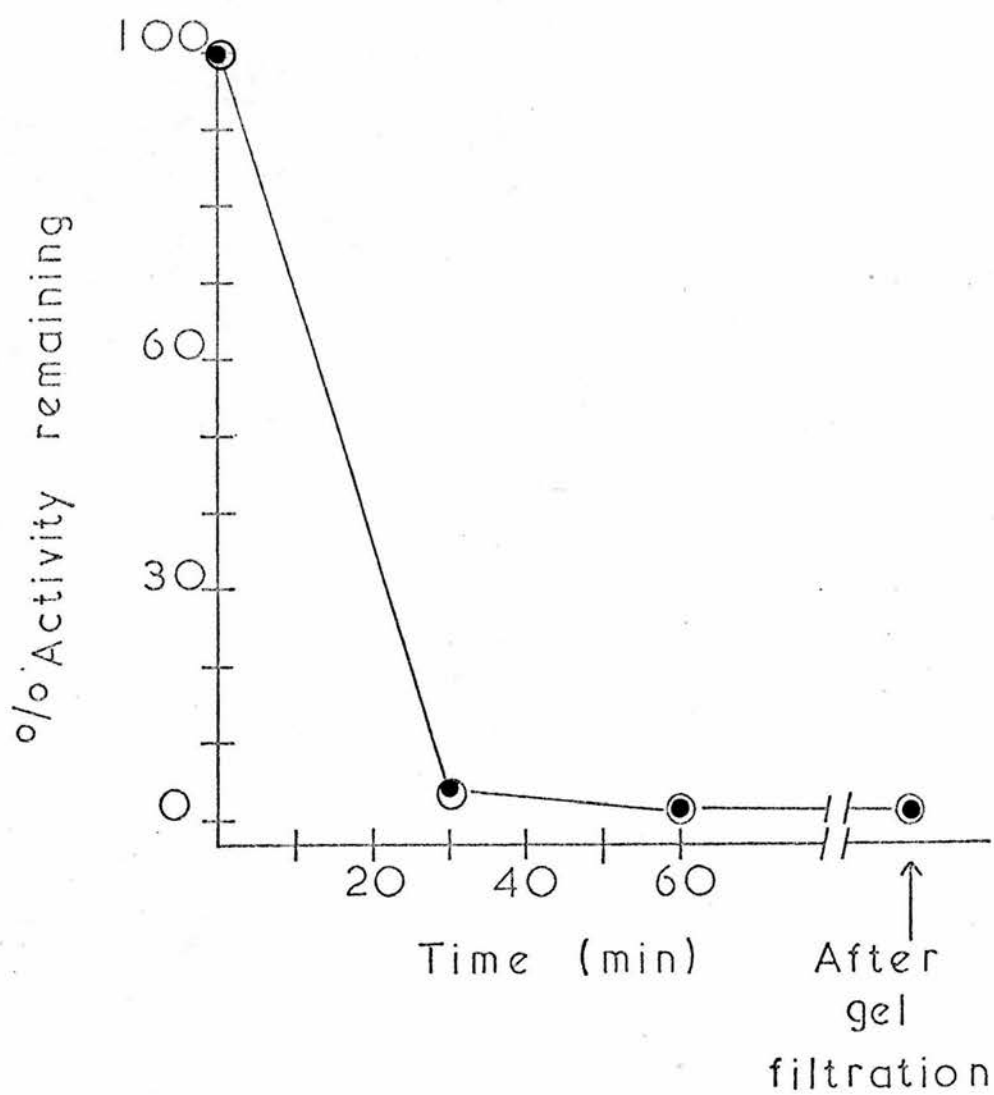


Fig. 73. Inactivation of a second large batch of pepsin C by DNM in two portions.

- - one portion
- - other portion

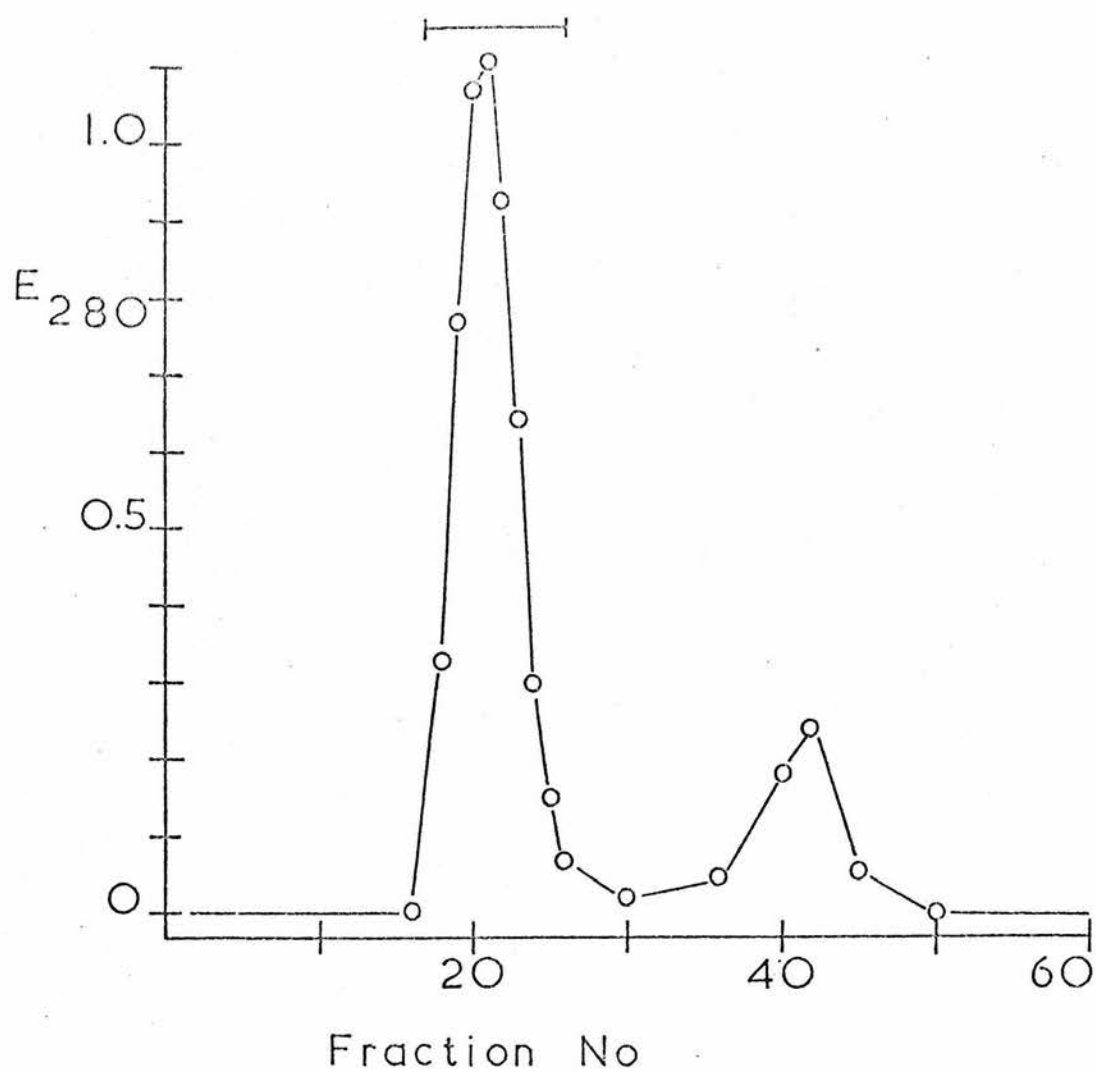


Fig. 74. Chromatography of the incubation mixture of pepsin C and DNM on Sephadex G-25 (8 x 50 cm), equilibrated with water.

14. The site of attachment of DNM in pepsin C (II).

It was necessary to see whether the aspartate (presumed to be) labelled in the sequence Ile-Val-Asp-Thr provided a carboxyl group necessary for the catalytic activity of the enzyme and to do this a 1:1 inhibited enzyme was essential.

14.1 Preparation of a second large batch of ^{14}C -inhibited pepsin C.

A new batch of radioactive DNM was prepared as before (Section 8.1) and amino acid analysis of the dipeptide ester before diazotisation showed glycine:norleucine::1:1.

Pepsin C (195 mg) was dissolved in 180 ml of 0.2M acetate buffer, pH 5.0, divided into two portions of 90 ml and each was placed in a cylindrical jacketed vessel at 20°. Cupric acetate (0.82 ml of a 0.1M solution) was added to each and after equilibration a solution of DNM (27 mg in 0.5 ml of methanol) was added to each. The variation in activity with time is shown in Fig. 73. After 60 minutes incubation the two portions were combined and applied to a column of Sephadex G-25 (8 x 50 cm), equilibrated with water. Fractions (40 ml) were collected and the elution profile is shown in Fig. 74. The fractions containing the inhibited enzyme were pooled as indicated by the bar and placed in an Amicon 'Diaflo' ultra-filtration cell, fitted with a UM 20 membrane. The volume was reduced to 80 ml overnight and this solution was freeze-dried. The yield obtained was 130 mg.

14.2 Calculation of the inhibitor content of inhibited pepsin C.

Approximately 2.5 mg of the inhibited enzyme was dissolved in 1 ml of water. Duplicate 0.05 ml samples were taken for scintillation counting and 0.1 ml was taken for haemoglobin assay of enzymic activity, which showed that the enzyme was completely inactive. The remainder of the solution was used for amino acid analysis.

Using the radioactivity of the inhibited enzyme to determine the inhibitor content, there were 1.20 moles of inhibitor per mole of enzyme. From the amino acid analysis there were 1.26 moles of norleucine per mole of enzyme, which agrees reasonably well with the former figure.

Although these analyses indicated that a 1:1 combination had not been achieved in this large batch either, the inhibitor content was sufficiently close to 1.0 to allow a reasonable certainty that, if a labelled peptide could be isolated from this batch, it was derived from a catalytically active group.

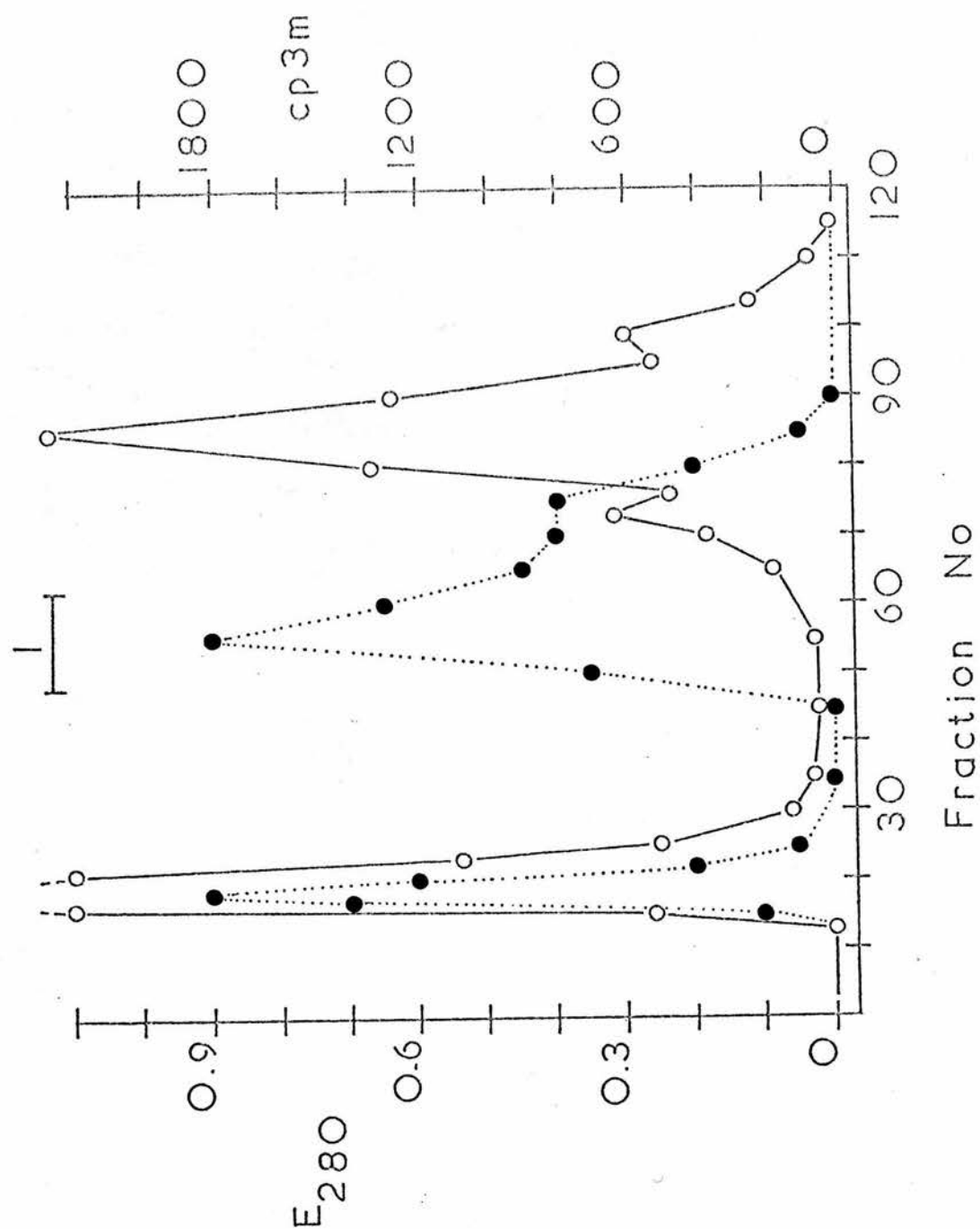


Fig. 75. Chromatography of the pepsin digest of inhibited pepsin C on Bio-Gel P 6 (3 x 52 cm), equilibrated with 5% acetic acid.

—○— - E₂₈₀
●..... - radioactivity

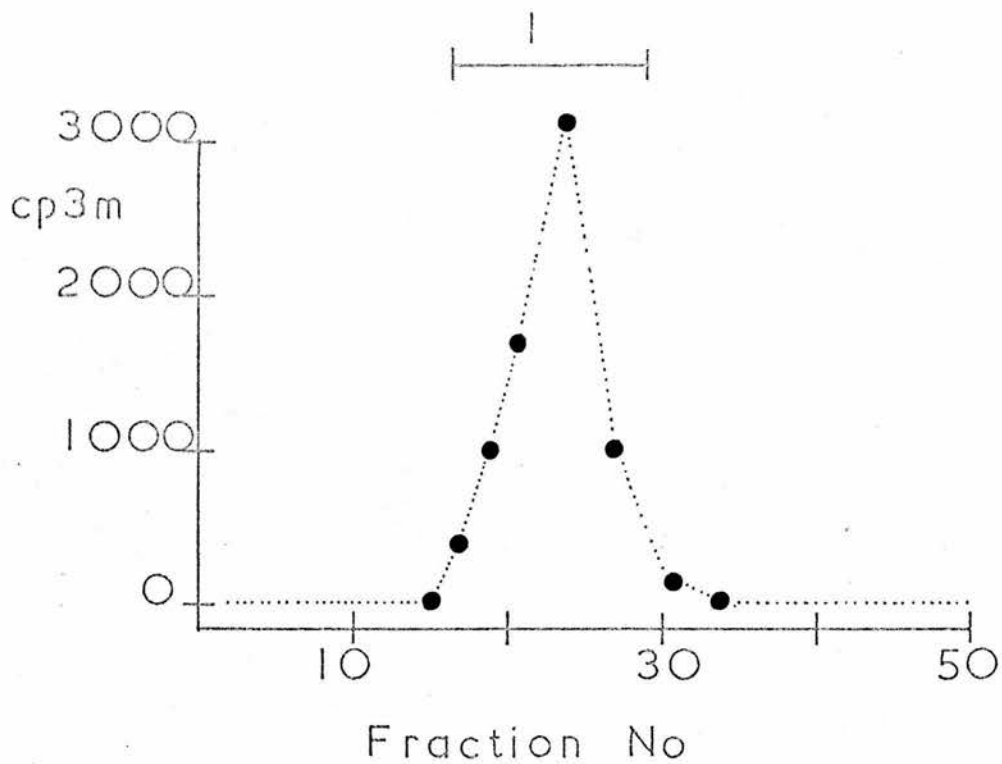


Fig. 76. Re-chromatography of fraction P1 on Bio-Gel P 4 (2 x 50 cm), equilibrated with 5% acetic acid.

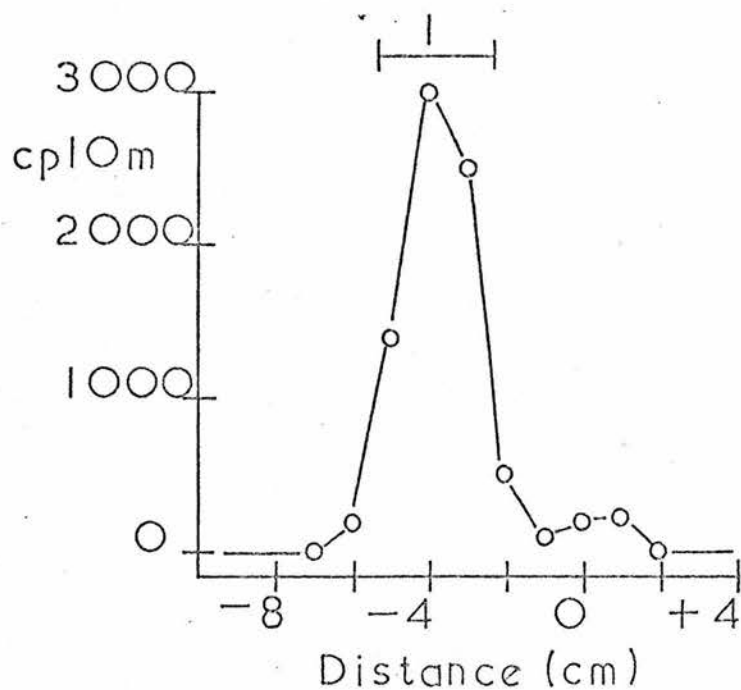


Fig. 77. Electrophoresis at pH 3.6 of fraction P1.

15. Degradation of inhibited pepsin C by pepsin.

The inhibited enzyme (130 mg) was dissolved in 15 ml of 0.02N-HCl. Pepsin (7 mg) was added and this mixture was incubated at 37° for 40 hours. The progress of the digestion was followed by taking samples for ninhydrin assay and these showed that pepsin had split one bond in eleven on average. At the end of the incubation, the insoluble material present was centrifuged off and washed with 5% acetic acid. Scintillation counting showed that 95% of the radioactivity was in the soluble fractions.

The insoluble material was discarded and the supernatant and washings were applied to a column of Bio-Gel P 6 (3 x 52 cm), equilibrated with 5% acetic acid. Fractions (5 ml) were collected and 0.2 ml samples were taken for scintillation counting. The elution profile is shown in Fig. 75. The material emerging at the void volume of the column was presumably undigested pepsin C and these fractions were pooled and freeze-dried. Fraction 1 was obtained as indicated by the bar. The recovery from the column was 89%.

15.1 Re-chromatography of fraction 1.

Fraction 1 was dried in vacuo, re-dissolved in 5% acetic acid and applied to a column of Bio-Gel P 4 (2 x 50 cm), equilibrated with 5% acetic acid. Fractions (2 ml) were collected and 0.1 ml samples were taken for radioactive assay (Fig. 76). There was no E₂₈₀ absorbing material present.

Fraction 1 was pooled as shown and the recovery of radioactive material was 87%. A sample of this material

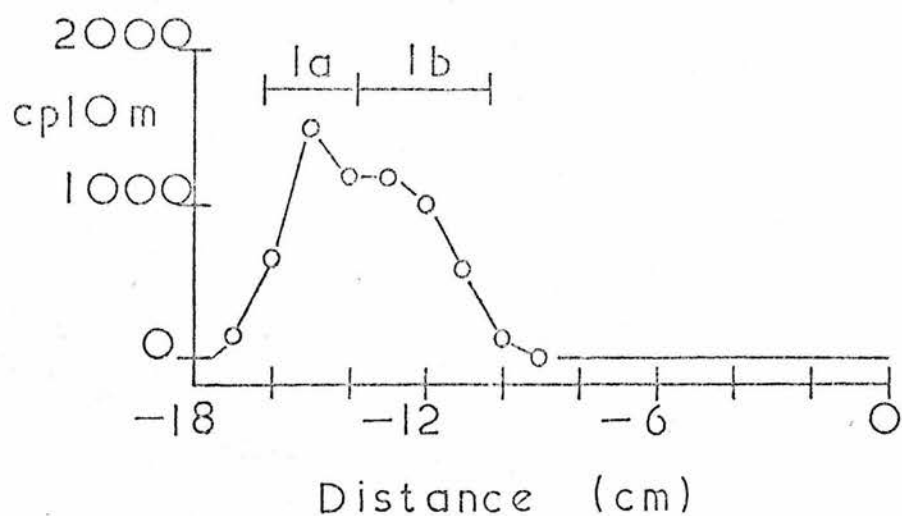


Fig. 78. Electrophoresis at pH 2.0 of P1 obtained from the pH 3.6 electrophoresis.

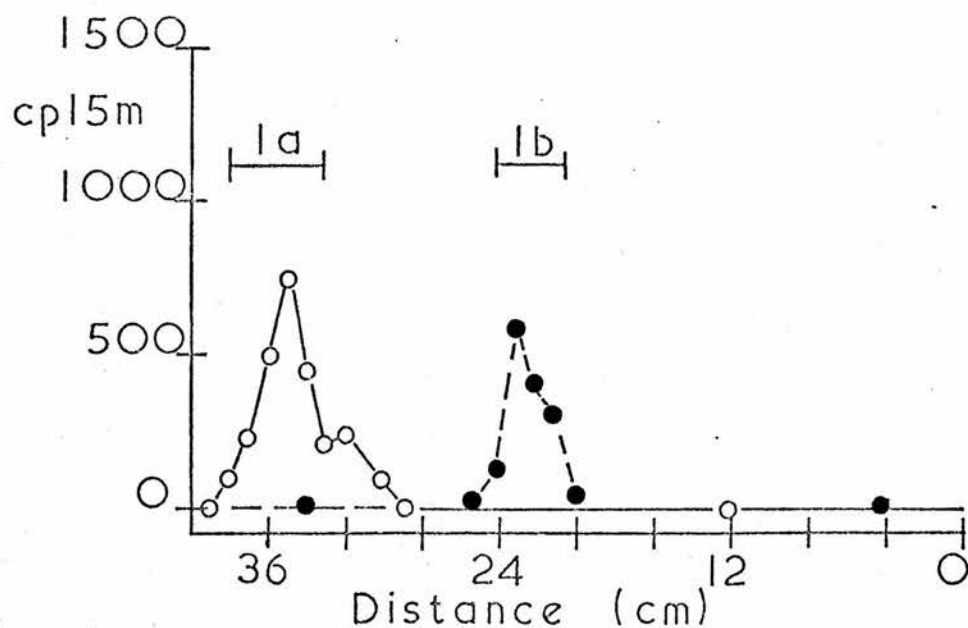


Fig. 79. Chromatography in t-butanol:methyl ethyl ketone: water::2:2:1 of Pla and lb, obtained from the electrophoresis at pH 2.0.

—○— - Fraction Pla
 -●- - Fraction Plb

was hydrolysed for 24 hours and amino acid analysis showed that further separation was necessary since the number of residues of the other amino acids was high in relation to norleucine.

a) Further separation of fraction 1.

This fraction was dried in vacuo, re-dissolved in 5% acetic acid and applied to No. 1 paper in a 4 inch strip. The paper was subjected to electrophoresis at pH 3.6 at 2 kV for 2 hours (Fig. 77). This radioactive material must be neutral at pH 3.6 as a neutral marker migrated identically to the labelled band. Band 1 was cut out, stitched into a fresh sheet and re-run at pH 2 (Fig. 78). This gave a partial separation of band 1 into two components, 1a and 1b. These were cut out separately, concentrated for paper chromatography as already described and run in t-butanol:methyl ethyl ketone:water overnight.

Unfortunately, it was found that the solvent appeared to have travelled about half-way down the paper and then stopped, leaving a yellow-brown line across the paper and the peptides had piled up behind this. This effect suggested that the solvent was evaporating from the paper, possibly due to non-equilibration. After many trial runs with blank papers it was found that the remedy was NOT to grease the lid of the chromatography tank. This was interpreted to mean that one (or more) of the solvents in the system was dissolving through the grease and continually removing the system from equilibrium. The yellow-brown band on the paper, isolated from a blank piece of paper, was

INHIBITED PEPSIN C

(130mg = 3.6 μ moles)

Gel Filtration on Bio-Gel P 6

HIGH MOLECULAR
WEIGHT MATERIAL
(50mg = 35%)

FRACTION 1
(1.4 μ moles =
40%)

LOW MOLECULAR
WEIGHT MATERIAL
(0.9 μ moles = 25%)

Rechromatography
on P 4

FRACTION 1

(1.2 μ moles = 33%)

HVPE at pH 3.6

BAND 1

(1.2 μ moles = 33%)

HVPE at pH 2.0

1a

1b

(0.65 μ moles = 18%)

(0.5 μ moles = 14%)

Paper chromatography

1a

1b

(0.15 μ moles = 5%)

(0.1 μ moles = 3%)

Balance Sheet for the purification of bands 1a and 1b.
(where possible, the yields have been found from both
isotope and norleucine contents and are in agreement)

eluted and hydrolysed and amino acid analysis showed that it was non-peptide material.

Bands 1a and 1b were re-run in this system without greasing the lid of the tank (Fig. 79). The R_F values (0.74 and 0.50 respectively) may not be truly representative because of this grease effect. ^{The balance sheet (opposite) shows that} rather large losses of material were incurred ^{at this stage.} Bands 1a and 1b were eluted from the papers with 5% acetic acid. Samples of 1a were hydrolysed for 24 and 72 hours and a sample of 1b was hydrolysed for 24 hours. Amino acid analysis gave the following results, relative to norleucine = 1.0:-

	Asp	Thr	Ser	Pro	Gly	Ala	Val	Ile	Leu	Nle	Phe
1a 24 hrs	1.0	1.0	-	-	-	-	0.8	0.8	-	1.0	-
1a 72 hrs	1.0	1.0	-	-	-	-	1.0	0.9	-	1.0	-
1b 24 hrs	1.0	1.8	1.0	0.7	1.0	0.5	0.9	0.7	0.7	1.0	0.3

The value for isoleucine in peptide 1a is a little low but peptides containing isoleucine, with paper chromatography as the final separation step, have been found to give low recoveries of isoleucine (Ambler 1963). It was not thought worthwhile to re-subject this peptide to electrophoresis after the paper chromatography step because of the low recovery achieved after the paper chromatography upset.

N-terminal analysis of 1a by DNS-Cl showed DNS-isoleucine. After one cycle of Edman degradation DNS-Cl showed the penultimate residue to be valine. Treatment of 1a with carboxypeptidase A released 0.93 μ moles of threonine per μ mole of peptide and nothing else. Peptide 1a must be:-

Ile-Val-Asp-Thr

The remainder of this peptide was used in an attempt to show whether the inhibitor was attached to the β -carboxyl group of the aspartate residue. The peptide was treated for 30 minutes on paper with triethylamine by allowing this to diffuse in from the edges of the band as in wetting a paper for electrophoresis. The paper was then subjected to electrophoresis at pH 3.6. The untreated peptide migrated -5 cm in accordance with its supposed neutral composition. The treated peptide migrated -0.5 cm (detected by ninhydrin) and no radioactivity could be detected anywhere on this strip. This presumably means that the norleucine derivative released by the triethylamine was soluble in the white spirit in the electrophoresis tanks.

This different mobility is in accord with the hydrolysis of an ester bond by the triethylamine, unmasking a carboxyl group which at pH 3.6 has at least a partial negative charge. This was taken as evidence that DNM reacts with a β -carboxyl group of an aspartate residue in the enzyme.

N-terminal analysis of lb by DNS-Cl showed DNS-isoleucine. After one cycle of Edman degradation, DNS-Cl showed the penultimate amino acid to be valine. After a second cycle, the new N-terminus was found to be aspartate. This gives an N-terminal sequence of:-

Ile-Val-Asp-

The remainder of the peptide was treated with carboxypeptidase A and the time course showed the release of 0.3 μ moles of phenylalanine and 0.5 μ moles of alanine per

μ mole of peptide. Leucine ($0.3 \mu\text{moles}/\mu\text{mole}$) was released very slowly as was serine ($0.2 \mu\text{moles}/\mu\text{mole}$) and threonine was released in parallel with the serine. These results can be explained by postulating that this fraction was heterogeneous at its C-terminus by having fractional residues of proline, leucine, alanine and phenylalanine

(cf. the amino acid analysis). It is not possible to deduce any sequence information from the carboxypeptidase data but the phenylalanine, alanine, leucine, serine and one threonine residue must lie near the C-terminus. Since this fraction has a homogeneous N-terminus, it can be formulated as:-

Ile-Val-Asp(Thr,Gly)(Thr,Ser)(Pro,Leu,Ala,Phe)

An overlap with peptide 1a suggests that the sequence may be:-

Ile-Val-Asp-Thr-Gly(Thr,Ser)(Pro,Leu,Ala,Phe)

16. Discussion

It has been shown that when porcine pepsinogen C is activated to pepsin C by incubation at pH 2, the activation peptides can be removed satisfactorily from the active enzyme by chromatography on SE-Sephadex. Pepsin C prepared by this method was found to be similar in its properties to the enzyme prepared by other methods. Amino-terminal analysis on the enzyme revealed the presence of 0.6 μ moles of serine and 0.2 μ moles of isoleucine or leucine per μ mole of protein. This was interpreted to mean that activation of the zymogen leaves a product with a slightly heterogeneous N-terminus, although it could also mean that the enzyme contained two polypeptide chains or that the preparation was contaminated with peptide(s).

Treatment of pepsin C and pepsinogen C with carboxypeptidase A released 2 moles of alanine and 1 mole each of phenylalanine, threonine, glycine and valine per mole of protein and this was taken as evidence that the C-terminal composition of both zymogen and enzyme was:-

(Val,Gly)(Thr,Phe,Ala,Ala)

It could be, of course, that these amino acids were released from more than one chain, such as would occur with contaminating peptide(s), but since the same amino acids were released from both zymogen and enzyme, this would have to mean that the contaminating peptide(s) was also present in the zymogen. Since the zymogen has been shown to have serine as its sole N-terminal residue, (Ryle 1960a) the idea of the enzyme consisting of two polypeptide chains is also made most unlikely.

The fact that both enzyme and zymogen do have the same C-terminal sequence indicates that activation of pepsinogen C to pepsin C, in common with other inactive precursor→active enzyme transitions, proceeds by removal of peptides from the N-terminal end of the molecule. A conformational change may also be involved, such as has been found for pepsin, but there is no evidence on this point.

Since the nature of the group(s) conferring the catalytic activity on pepsin C had to be investigated by affinity labelling, attempts were made to synthesise chloro-acetyl leucine methyl ester and bromoacetyl leucine ester as possible pseudo-substrates which might interact with the assumed carboxyl group(s) in the active site of the enzyme.

However, it is very likely that cyclisation occurred under the alkaline conditions used for the preparation of both chloroacetyl leucine and bromoacetyl leucine so that neither CALOMe nor BALOMe were really synthesised. Thus, it is not possible to say how these compounds affect the activity of pepsin C.

Pepsin C has a preference for aromatic and long-chain aliphatic amino acids (Ryle, Leclerc & Falla 1968) so it was thought that diazoacetyl norleucine methyl ester (first used by Rajagopalan et al (1966b) for the inhibition of pepsin) would be a suitable substrate analogue to try with the minor enzyme (although Lunblad & Stein (1969) have since shown that the specificity of the reaction of the diazoacetyl compound with pepsin does not depend on the side-chain of the amino acid used). Norleucine was used rather than leucine or isoleucine as it is an unnatural amino acid and can be

estimated easily in the presence of the branched chain isomers on the amino acid analyser.

Although Fruton et al (1939) have shown that peptic hydrolysis of model dipeptide substrates requires absolutely that the amino acids be of the L configuration, it was not possible to use diazoacetyl-L-norleucine methyl ester in the experiments as this could not be made to crystallise. The racemic mixture, on the other hand, crystallised fairly readily and this was used rather than the L isomer.

The inhibition experiments with DNM showed that this compound would inactivate pepsin C but the degree of inactivation varied considerably with the conditions used. In agreement with other authors (Zollinger 1961; Rajagopalan et al 1966b), it was found that cupric ions were essential for 'activation' of the diazo compound since DNM had very little effect on the enzyme in the absence of copper. The metal ion may induce formation of a metal complexed carbene intermediate (Yates 1952) which could interact with a protonated carboxyl group in the enzyme. Other metallic ions were not tried in this system but it has been found (Lundblad & Stein 1969) that Ag^+ but not Cd^{++} , Co^{++} , Pb^{++} , Zn^{++} or Ni^{++} (Kozlov, Ginodman & Orekhovitch 1967) can function in place of Cu^{++} with the analagous inhibition of pepsin by diazo compounds.

Inactivation was achieved much more readily at pH values above 4.5 and this was assumed to mean that at lower pH values the highly reactive diazo group was destroyed by the acidity of the medium before it could interact with the enzyme. Lundblad & Stein do not subscribe to this view.

They propose that the positively charged copper carbene complex is bound at a negatively charged carboxyl group and then reaction can occur at a neighbouring protonated carboxyl group. This was proposed because diazo compounds, which are thought to react via a carbene intermediate (Kirmse 1964), have been shown to have a preference for protonated carboxyl groups (Roberts, Watanabe & M^CMahon 1951; Doscher & Wilcox 1961). The two carboxyl groups postulated for pepsin, one with a pK_a of around 1 and the other with a pK_a of about 4, should both be negatively charged around pH 5 and so could bind the positively charged reagent. It was thus necessary to postulate the existence of a third (protonated) carboxyl group with a higher pK_a with which the reagent would then react. This hypothesis does not really explain the lack of reaction at low pH values, since the proposed carboxyl group with pK_a of 1 should be mostly ionised at pH 2 and could therefore bind the carbene intermediate which could then react with the carboxyl group with pK_a of 4, since it ought to be fully protonated at this pH value.

Whichever of these explanations applies, the fact remains that the incubation had to be carried out above pH 4.5 to produce complete inactivation and all subsequent incubations of enzyme and inhibitor were carried out in acetate buffer, pH 5.0.

The optimum conditions for complete inactivation were found to be at enzyme/cupric ions/DNM molar ratios of 1/30/40 but by varying the amount of inhibitor added it was

possible to obtain enzyme inhibited to varying extents. Analysis for the inhibitor content showed that the inactivation was proportional to the extent of incorporation of inhibitor into the enzyme, up to complete inactivation at reaction with one mole of inhibitor per mole of enzyme. Thus, although the diazo compound is a highly reactive species, specific labelling of one group in the enzyme is apparently being achieved (bearing in mind the partial reaction of two histidine residues in ribonuclease with iodoacetate - Crestfield, Moore & Stein 1963b). Examination of the data to determine the dependence of the rate of inactivation on the inhibitor concentration showed tentatively that the inactivation proceeded through the formation of an intermediate E-I complex with an apparent dissociation constant of about 0.2 mM. Such an intermediate is to be expected if inactivation is occurring by specific reaction at the active site.

It is impossible to say definitely whether this group being attacked is part of the active centre without any kinetic information from pH-rate profiles, binding strengths etc. but from the evidence of the stoichiometric incorporation and concomitant loss of activity, the group is uniquely reactive towards DNM and is certainly involved in the catalytic activity of the enzyme. Further experiments were done to try to clarify this problem.

APD, which has been shown to be a competitive inhibitor of pepsin C at pH 2, was found to protect the enzyme, to some extent, from inactivation by DNM and this suggests that the

irreversible inhibitor is bound at the active site prior to formation of a covalent bond with the enzyme.

Incubation of DNM with denatured pepsin C which, by definition, must have its active centre disrupted, led to a very low level of incorporation of inhibitor into the enzyme, also suggesting that the inhibitor binds in the active centre in the native enzyme.

When pepsinogen C, which, as the inactive precursor, ought to have the active site masked (or not in its native conformation) was incubated with DNM rather more complicated results were produced. Incubation at pH 5 produced varying degrees of inhibition and it was found that the zymogen slowly precipitated at this pH, thus complicating the removal of excess of inhibitor by chromatography on Sephadex. When the pH of the mixture was taken to 5.6 at the end of incubation the zymogen chromatographed quite normally, which would seem to indicate that pH 5.0 is too close to the isoelectric point to allow complete dissolution of the zymogen. In this incubation (Section 7.10) the zymogen lost about 65% of its potential activity with concomitant incorporation of 0.65 moles of inhibitor per mole of protein. A control incubation revealed that there was very little (ca 5%) activation of the zymogen to pepsin C. When pepsinogen C was incubated with DNM at pH 7, only 10% of the potential activity was lost and 0.1 residues of inhibitor were incorporated. This could indicate that at pH 7 either the reactive -COOH is -COO^- which is not supposed to interact with the copper complexed intermediate or that the zymogen

has a conformation which does not have the active site in its native form (or masked) and on lowering the pH to 5 the zymogen begins to refold so that the active site is beginning to exist in the native form but without release of the activation peptides (which needs a higher strength of acidity) which would form the active enzyme. Thus, the activation peptides could protect the active site to some extent so that the zymogen only undergoes a partial inactivation where the enzyme is fully inactivated. This hypothesis obviously requires further study but it raises the point of whether the active site of pepsin C has the same conformation at pH 5 where the inactivation is carried out and at pH 2 where the enzyme exerts its maximum activity and hence whether the group being labelled at pH 5 is involved in the catalysis at pH 2.

Jackson et al (1969) studied the hydrolysis of acetyl-phenylalanyl-tyrosine and its ester and amide by pepsin over the pH range 2-5 and found kinetic evidence that there was no large change in conformation of the active site over this pH range. Since pepsin C is very similar in its properties to pepsin, by analogy this might be extended to cover pepsin C. Indirectly, the question was also answered since, although the incubation of the enzyme with DNM was carried out at pH 5, measurement of the degree of inactivation (i.e. loss of activity vs haemoglobin) was carried out at pH 2. It can be proposed that, since the ability to digest haemoglobin at pH 2 was lost, the group labelled at pH 5 is involved in the catalysis at pH 2.

The E-I derivative, once formed, also appears to be stable at low pH values.

As a result of these experiments it can be stated that DNM interacts with a group in the enzyme with a concomitant loss in activity and, although it cannot be said categorically that this group occupies the active site of the enzyme, indirect evidence suggests that (one of) the catalytically important group(s) may well be involved in this locus.

It was obviously of interest to characterise the site of attachment of the inhibitor by finding its position in the amino acid sequence. A large batch of inhibited enzyme was thus necessary to provide sufficient starting material for the protein chemistry studies and to facilitate separation of the peptide(s) from the inhibitor binding site, radioactive DNM was used. The inhibitor was thus doubly labelled i.e. in the diazo portion by means of the radioisotope and in the C-terminal portion since norleucine, an unnatural amino acid, was being used.

The preparation of the inhibited enzyme was carried out under standard conditions of incubation except that the enzyme concentration (and therefore also the cupric ion and DNM concentrations) was increased from 1.2 to 1.7 mg/ml to minimise the large volume of enzyme solution to be handled. Unfortunately, it was found that 3 moles of inhibitor had been incorporated per mole of enzyme instead of one. It is suggested that this might be due to an enzyme catalysed incorporation of a second (or third) residue of inhibitor

into a second molecule of the E-I complex and this would be more important at higher concentrations of enzyme, since an $[E][E-I]$ term would be involved.

This large batch of inhibited enzyme was too valuable to waste, however, and it was thought that it might be useful, if the 3 sites of attachment could be found, to compare these with the site of attachment of a 1:1 inhibited enzyme, since it has been suggested that the active site of pepsin contains two (or possibly three) carboxyl groups and the carboxyl group found to be labelled by p-bromophenacyl bromide (Erlanger et al 1967) occurred in a different peptide sequence from that found to be labelled by 1-diazo-4 phenyl butan-2-one (Hamilton et al 1967) and later that labelled by diazoacetyl phenylalanine methyl ester (Bayliss et al 1969).

After preliminary experiments to find the optimum conditions for digestion of the inhibited protein and separation of the labelled peptides, subtilisin was selected as the enzyme most likely to give small easily-identifiable fragments. However, it proved very difficult to isolate labelled peptides from the large-scale subtilisin digest. The peptides:-

Val-Asp

and Leu-(Ile,Val,Asp)

were obtained but most of the radioactive label was contained in fractions which had only one amino acid, norleucine. This suggests that the enzyme-inhibitor bonds were cleaved under the conditions used for the digestion or the series of

separations (or both). It was found that norleucine methyl ester was readily saponified to the free carboxylic acid at pH 8.5, the pH at which the subtilisin digest was carried out. Since the inhibitor was supposed to react with a carboxyl group(s) in the enzyme through the formation of an ester bond(s), this observation might explain why most of the inhibitor residues were found as inhibitor breakdown products.

Thus, it was necessary to keep the conditions of digestion and separation acidic to prevent hydrolysis of the enzyme-inhibitor bonds. The two most common methods for protein fragmentation under acidic conditions are cleavage with cyanogen bromide and pepsin. These techniques were applied to the inhibited pepsin C and it was possible to isolate from a peptic cleavage, the labelled peptides:-

Ile-Val-Asp-Thr

and Ile-(Val,Asp,Thr,Gly,Thr)-Ser

If it is assumed that these are derived from the same labelled site, as seems likely, then the labelled sequence:-

Ile-Val-Asp-Thr-(Gly,Thr)-Ser

can be established by overlapping. This was the only labelled sequence which could be found from the peptic digest.

The subtilisin peptides fit into this scheme very well, if the assumption is again made that they are derived from the same labelled site. Thus, the sequence:-

Leu-Ile-Val-Asp-Thr-(Gly,Thr)-Ser

can be established.

When the pilot cyanogen bromide digest indicated that one third of the total radioactivity, after fission, was soluble in 5% acetic acid, it was thought that this might be a useful technique to employ to find one of the sites of attachment and the other two sites might be found from a peptic digest of the insoluble material. However, when the cyanogen bromide fission was carried out on a larger batch of inhibited enzyme, electrophoresis of the soluble fraction revealed that the radioactive material was almost equally divided among about six bands. One of these bands was found to contain only norleucine and the amino acid compositions of some of the others indicated that the norleucine was not attached to an acidic residue in the peptides. Since DNM was used as an inhibitor with the specific aim of modifying a carboxyl group(s), these results suggest that the radioactive material in this soluble fraction was probably some derivative(s) of norleucine i.e. inhibitor which had become detached from the protein, contaminated with unlabelled peptides.

Peptic cleavage of the insoluble material from the cyanogen bromide fission produced one fraction which was almost homogeneous. To explain the observed results it had to be postulated that this fraction was a mixture of two peptides:-

Ala-(Gly,Thr,Ser,Leu,Ile,Val,Asp,Thr,Gly,Thr,Ser)-Pro-Ala-Leu
and Ile-(Val,Asp,Thr,Gly)-Thr-Ser

This hypothesis was based on the assumption that this labelled fraction was derived from the same inhibitor binding

site as the

Leu-Ile-Val-Asp-Thr-(Gly,Thr)-Ser

sequence which has been established. This fraction could, of course, be derived from another site but the similarity in amino acid composition to the established sequence suggests that this is not so. Not nearly enough information was obtained to be sure that the sequence proposed is correct but if the proposal is tentatively accepted, then the labelled sequence can be extended to

Leu-Ile-Val-Asp-Thr-Gly-Thr-Ser-Pro-Ala-Leu

This over-all peptide contains 10 neutral amino acids and the one ionizable side-chain is (presumably) modified by the neutral inhibitor residue; this might explain why isolation of the labelled peptides proved so difficult.

It was not possible to isolate any other labelled sequence from this batch of 3:1 inhibited enzyme even when degradation was carried out under acid conditions. Indeed, several fractions were purified from the peptic digest which proved to contain only norleucine. This must mean that two of the enzyme-inhibitor bonds are very labile even under acid conditions.

Dr. J.R. Knowles (personal communication) found that with his analagous work on pepsin, where a 1:1 inhibited enzyme was being used, most of the inhibitor was released if the pH was not kept below about pH 7, whereas there was no release if the pH was kept below this value. With this in mind, it is suggested that the bond between the inhibitor molecule and the Ile-Val-Asp-Thr sequence is the important

link between the inhibitor and an active site group whereas the other two (very labile) enzyme-inhibitor bonds are of minor importance in the loss of enzymic activity, particularly since it has been shown that the inhibited enzyme, once formed, cannot be re-activated by standing at pH 2.

To prove this absolutely it was necessary to prepare a batch of 1:1 inhibited enzyme and show whether the site of attachment did reside in the Ile-Val-Asp-Thr sequence. When the enzyme, at a concentration of 1.2 mg/ml, was incubated with the inhibitor, it proved possible to isolate fully inhibited enzyme containing 1.2 inhibitor residues per mole of enzyme. This was slightly higher than had been hoped for but since incubation had been continued for 30 minutes after almost complete inactivation had been attained, the additional 0.2 residues could be due to non-specific incorporation into the inhibited enzyme.

Since pepsin had proved to be the most useful enzyme for degradation of the previous batch this new batch of inhibited enzyme was also fragmented with pepsin. Once again the peptide:-

Ile-Val-Asp-Thr

was isolated, this time without any sign of any inhibitor breakdown derivatives. It was proposed that the other labelled fraction which was isolated was heterogeneous at its C-terminus by having fractional residues of proline, leucine, alanine and phenylalanine i.e.

Ile-Val-Asp(Thr,Gly)(Thr,Ser)(Pro,Leu,Ala,Phe)

If this is the case, then the sequence:-

Ile-Val-Asp-Thr-Gly (Thr, Ser) (Pro, Leu, Ala, Phe)
1 5 10

can be established by overlapping with the Ile-Val-Asp-Thr peptide. The total amount of these peptides recovered (before the final chromatography step - Section 15.1a) was much higher than could have been achieved from the additional 0.2 residues of inhibitor. The above sequence can be extended by comparison with the

Ile-Val-Asp-Thr(Gly,Thr)Ser
sequence already established, to:-

Ile-Val-Asp-Thr-Gly-Thr-Ser(Pro,Leu,Ala,Phe)

It can be said that the inhibitor is attached to the β -carboxyl group of the aspartate residue in this sequence because:-

- a) the aspartate (along with valine) is the only common residue among the peptides isolated and reaction with the valine side-chain seems unlikely
- b) in several peptides the aspartate is not C-terminal, excluding reaction with the α -carboxyl
- c) the apparent neutrality of the labelled peptides (from their migration on electrophoresis)
- d) the base lability of the enzyme-inhibitor bond suggests an ester bond
- e) loss of the inhibitor residue from the peptide produces a peptide with a partial negative charge on electrophoresis at pH 3.6.

Thus, it can be concluded that DNM reacts specifically with the β -carboxyl group of an aspartate residue in the sequence:-

Ile-Val-Asp-Thr

and this can be tentatively placed in the sequence:-

Leu-Ile-Val-Asp-Thr-Gly-Thr-Ser (Pro, Leu, Ala, I₁₆)

A mild acid hydrolysis on this sequence would have been very useful in placing the doubtful residues 6-11 but it did not prove possible to isolate the appropriate peptides in large enough quantities to make this feasible.

This sequence for an active site group of pepsin C contains an identical fragment to that proposed for pepsin (i.e. Ile-Val-Asp-Thr-Gly-Thr-Ser) by Bayliss et al (1969) and (Ile-Val-Asp-Thr) by Fry et al (1968).

The

homology in the two active sites is very interesting in view of the fact that, although both enzymes have similar specificities with regard to the amino acids in the bond to be split (Ryle & Porter 1959), pepsin has activity towards synthetic substrates (e.g. APD) where pepsin C does not.

The minor enzyme's inability to hydrolyse the peptide substrate could be due to a number of factors, e.g. it could be that, in an analagous way to lysozyme, the enzyme may require the substrate to have a long chain of amino acid residues before it can carry out the hydrolysis.

In view of the homologies found between trypsin, chymotrypsin and elastase with regard to their amino acid sequences, active sites and over-all conformations (Shotton & Hartley 1970), it is perhaps not surprising that two similar enzymes like pepsin and pepsin C should have identical sequences around (one of) the reactive carboxyl residue(s). Presumably the difference in activities towards peptide substrates is due to difference(s) in the three-dimensional conformations of the molecules or in amino acid sequence elsewhere than at the active site. Complete homology has been established for the active sites of pepsin and pepsin C, some homology ^{may} exist in the C-terminal regions and it has been shown (Falla & Ryle unpublished observations) that some homology, but not a great deal, exists between the peptides produced on activation of pepsinogen C to pepsin C and pepsinogen to pepsin. Speculating (certainly very dangerously) on the limited amount of sequence data available from pepsin C and pepsin, it would seem that there is not as much homology between these two enzymes as between chymotrypsin and trypsin which, although only possessing 40% homology over-all, show about 85% homology when the reactive interiors of the molecules are considered. The differences in specificity of trypsin

and chymotrypsin have been found to be, not at the 'gross' level of three-dimensional conformation but simply by inter-change of an aspartate and serine residue in position 189 in the sequence (i.e. 6 residues removed from the active site serine 195). The specificity of trypsin for dibasic amino acids can thus be explained by the negatively charged aspartate residue attracting the positively charged lysine and arginine residues into the specificity site of the enzyme. (This leads to speculation on whether an analagous enzyme with either a lysine or arginine in position 189 would be specific for aspartate and glutamate residues. It would certainly be a useful tool for protein chemists if it existed or could be prepared.)

Thus it may be that pepsin C and pepsin differ, with respect to their abilities to hydrolyse APD, in a rather more 'gross' conformational alteration than an amino acid exchange such as with trypsin and chymotrypsin. This is obviously an oversimplification of the problem since no account has been made for the abilities of the enzymes to bind peptides, as was mentioned earlier. It is hoped that in the future, synthetic peptide substrates may be designed for pepsin C and further deliberation on this point would be better deferred until more is known about the ability of pepsin C to hydrolyse peptides.

It is rather difficult to see any advantage to the system in retaining both of these enzymes since both have very similar activity towards protein substrates and the major enzyme has activity towards small synthetic peptides. If the minor enzyme, rather than the major enzyme, had possessed the 'peptidase' activity in addition to protease activity it could be understood why the pig had maintained small but discreet levels of minor enzyme in order to degrade small peptides which might occur among the protein in the diet of the animal. It may be that the minor enzyme

is present in porcine stomachs to degrade some material (possibly an oligopeptide with some peculiarity of structure which might make it resistant to peptic digestion) normally found in the staple diet of the pig. Since the pig is now a domesticated animal and might have evolved this enzyme to cope with this hypothetical peptide in its human-provided diet (although the time scale is probably not long enough for this), it would be interesting to see whether a wild boar from some remote corner of the earth had the genetic complement for both major and minor enzymes. This problem is not peculiar to the pig since other species such as cow, chick and human have been shown to have some heterogeneity with regard to their gastric enzymes and in some cases, such as the dogfish - certainly not a domesticated animal - a zymogen has been isolated which is very similar to porcine pepsinogen C. Therefore any explanation for the heterogeneity must encompass these other species as well.

In conclusion, it has been shown that inactivation of pepsin C by DNM specifically labels the β carboxyl group of an aspartate residue in the sequence Ile-Val-Asp-Thr, which it is suggested provides (one of) the active site residue(s) of the enzyme and this is identical to the system for pepsin.

There are some other aspects of this work which ought to be looked at. The primary objective must obviously be to develop a suitable synthetic peptide substrate for pepsin C which would permit kinetic studies on binding constants, catalytic rate constants and variations in these with pH and alteration of the groups involved in the substrate. From

this it could be found whether pepsin C is analagous to pepsin in involving carboxyl groups in the active centre, enhancing the affinity labelling studies.

These could also be extended by modification of the irreversible inhibitor. Diazoacetylglycinamide might be tried to see whether there is any specificity for the side-chain of the amino acid in the inhibitor and whether this inhibitor could give rise on occasion to an inhibited enzyme containing more than one residue of diazoacetylglycinamide per mole of protein. Could these additional binding sites be identified? It would also be interesting to delve more deeply into the actual mechanism of interaction of diazocompounds with pepsin C, particularly in view of the need to include cupric ions and to carry out the incubation above pH 4.5. A rather different irreversible inhibitor with the diazo group at the C-terminal rather than the N-terminal end, perhaps something like Z-Phe-Phe.CHN₂ or diazoacetyl-Phe-Phe-OMe, might be closer substrate analogues which could shed further light on these problems.

Finally, a more detailed study on the interaction of pepsinogen C with diazo compounds might be rewarding, to see whether any conformational changes are involved in the zymogen over the pH range 2-7, how the inhibitor binds to the zymogen, how many residues are bound and if so, what the sites of attachment of the inhibitor to the zymogen are and how do these compare with the active site peptide found for pepsin C in this thesis.

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